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EFFECT OF FERMENTATION WITH ASPERGILLUS ORYZAE NRRL506 AND ASPERGILLUS JANUS NRRL1935 ON THE NUTRITIONAL VALUE OF COTTONSEED MEAL

Iowa State University PH.D. 1980

Effect of fermentation with *Aspergillus oryzae* NRRL506 and *Aspergillus janus* NRRL1935 on the nutritional value of cottonseed meal

by

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GENERAL INTRODUCTION

Many conventional vegetable protein sources used in monogastric nutrition are deficient in one or more essential amino acids. Protein is an expensive commodity that it is not always economical nor beneficial to feed in excess to overcome amino acid deficiencies. Overcoming amino acid deficiencies in a diet by combining various vegetable sources is inadequate, cumbersome and involves a lot of inventory. Animal protein sources that are more balanced in their amino acid profile are usually relatively expensive. Hence, there is a need to search for other high quality sources to improve both the quality and quantity of the existing vegetable proteins.

Fungi are microorganisms which have the capability for improving vegetable proteins (Chah et al., 1976a). The use of fungi as food and in food processing dates back to early history in China (Hesseltine, 1965). Fungi produce protein of high quality and digestibility. Their production is not as dependent on the uncertainties of weather and human frailties as is conventional agriculture. They produce foods which are rich in protein and numerous vitamins. They have a very short generation time and can thus double their masses in about 4 to 12 hours (Kihlberg, 1972).

Conventionally, microbial proteins and amino acids are recovered from microbial culture in two ways. Microbial cell

material (bacterial and yeast proteins) is best removed by centrifugal separation but filamentous organisms are separated from a liquid culture using rotating filters on which the mycelium forms a felt or mat. Amino acids are recovered by adsorption on cation ion exchange resins (Rose, 1961). Any of these product recovery methods is not applicable to solid state fermentation with flimaentous fungi because the mycelium intertwines with the solid medium. Hence, the option left is to feed the fungal mycelium with the substrate from which it obtains its nutrients. In many fungus fermented foods, such as tempeh consumed in the Orient (Hesseltine, 1965), Roquefort cheese and blue cheese (Christensen, 1969), the mold remains as part of the basic food. Fungi alter organic components of foods to obtain energy for their growth (Pederson, 1971). They synthesize certain nutrients useful to man from the food ingredients and these nutrients may accumulate in the protoplasm of fungal cells or may be released into the food when the cell is autolyzed. The fungal cells generally constitute a small portion of the total mass of fermented food, even when millions of organisms are present at some stage in the fermentation. Nevertheless, nutritionally and flavorwise, they are important (Pederson, 1971).

The term mold or fungi has been most often associated with deterioration of stored grains and the concurrent production of substances that are toxic to animals (Beuchat, 1978). Hence, the potential use of nontoxic fungi for

improving the nutritional quality of animal feedstuffs has not received much attention.

The objectives of the research reported here were: (a) to develop and refine a procedure for solid state fermentation of cottonseed meal with Aspergillus oryzae NRRL506 and Aspergillus janus NRRL1935, (b) to evaluate the feeding potential of fermented cottonseed meal for broiler chicks and (c) to stimulate fungal production of amino acids through enriching cottonseed meal with ammonium sulfate. Cottonseed meal was selected for fungal-fermentation because information about products derived from it is scanty. Also, cottonseed meal has protein of poor quality, is high in crude fiber and contains some gossypol. Thus, there seemed to be ample opportunity for beneficial changes in the nutritional properties of cottonseed meal as a result of various biomodifications that may occur during fermentation with selective fungi.

GENERAL LITERATURE REVIEW

Potentials and Problems of Cottonseed Meal

Cottonseed meal (CSM) is a by-product of cottonseed oil extraction. Among the oil seed meals, it ranks second to soybean meal in abundance. It represents the most inexpensive source of protein for poultry feeding in many subtropical and tropical areas where production of cottonseed is extensive. In the United States, the production is confined to the southern region. The protein content of cottonseed meal varies from 36 to 50% depending on the method used to extract the oil (Phelps, 1966). Total protein and fiber content of cottonseed meals tend to be inversely related.

Cottonseed meal has a number of undesirable characteristics, each of which will be reviewed in order of importance as it affects broiler production.

Amino acids

Lysine is usually the first limiting amino acid in CSM followed by methionine, threonine, isoleucine and leucine (Phelps, 1966). Anderson (1965) stated that lysine was the most limiting amino acid when CSM was fed to chicks, and methionine was the second limiting amino acid. Several studies have shown that when lysine and methionine were supplemented to CSM diets, weight gains were close or equal to those obtained on soybean meal diet (Grau, 1946; Heywang and

Bird, 1950).

Energy and fiber

Energy content of cottonseed meals is inversely related to its fiber content (Phelps, 1966). Cellulosic materials constitute about 30% of CSM, and this cellulose portion is unavailable for nutritive purposes to monogastric animals due to the animal's inability to digest cellulose (Noyes, 1969). Naber and Morgan (1957) reported that 44% protein CSM contained 2 to 3 times as much fiber as 44% protein soybean meal. The National Research Council (NRC, 1977) indicated that solvent extracted CSM contained 2400 kcal metabolizable energy (ME)/kg and 13.6% crude fiber. Phelps (1966) reported 1600 to 2000 kcal ME/kg and 10 to 14% crude fiber in 41% protein CSM, and commented that there was a considerable variation in oil, fiber, moisture and protein quality of cottonseed meals. Therefore, significant differences in energy contents would be expected. Naber and Morgan (1957) reported that when high levels of CSM were used in a diet, energy was limiting. This limitation could be corrected by white grease supplementation. White grease (2 to 4%) was included in a 20% protein diet containing 34% CSM and 2% fish meal. Growth and feed efficiency of broiler chicks were equal to those of broiler chicks fed a soybean meal basal diet.

Gossypol

Gossypol is a highly reactive, polyphenolic, dinaphthaldehyde compound. Gossypol is found in cottonseed kernels where it is interspersed with pigment glands (Finlay et al., 1973). During processing of cottonseed, most of the free gossypol was removed by solvent extraction or detoxification. Detoxification was achieved under the influence of moisture and heat by condensation of aldehyde groups of gossypol with free amino groups of the protein to form bound gossypol (Clark, 1928). Cottonseed meal usage in most rations for non-ruminants has been influenced more by fiber, energy and amino acids than by gossypol levels (Smith, 1970).

Gossypol content and protein quality of CSM depend on the method used to extract cottonseed oil. The screw press method or expeller process was used to process approximately 40% of the 3 million tons of CSM produced in U.S.A. (Smith, 1973). The screw press method generates high temperatures. The high temperatures, generated during extraction of the oil by the screw press method, produced a meal low in free gossypol and low in protein quality. Protein quality was measured by protein solubility in dilute alkali or by determination of lysine with a free epsilon amino group (Phelps, 1966). About 30% of total U.S. production, however, consisted of prepress solvent meals which were usually low in free gossypol and relatively high in protein quality. The data shown in Table 1 (Morgan and Willimon, 1954) describe the gossypol content of

Table 1. Crude protein, fat and gossypol content of CSM

Product	Protein (%)	Fat (%)	Free gossypol (%)	Total gossypol (%)
CSM-solvent	41.0	1.64	.041	.38
CSM-screwpress	42.0	3.58	.029	.70
CSM-hydraulic	41.63	8.32	.064	.90

CSM prepared by various methods.

Smith (1970) reported that the low, free gossypol level in screwpress and prepress solvent meals was not a significant factor in determining the use of CSM in practical swine and poultry rations. The upper level of CSM for swine and poultry rations was determined by lysine, fiber and energy contents of CSM.

If gossypol were present in any substantial amount, the effect could be deleterious. Several workers have reported the adverse effect of gossypol from various sources on animals. Finlay et al. (1973) reported that the cottonseed kernel was internally interspersed with diaphthaldehyde, a substance that had a deleterious physiological effect on single stomach animals, such as poultry. Conkerton and Frampton (1959) described the mechanism of reaction of gossypol. Gossypol inhibited autocatalytic conversion of pepsinogen to pepsin. Apparently, the pepsinogen bridged

between two carbonyl groups of the gossypol molecule and two epsilon amino groups of the amino acid lysine.

Skutches et al. (1974) fed pigs diets that contained .06% free gossypol. The blood samples from the pigs indicated reduced hemoglobin and hematocrit levels. Liver iron concentration was reduced, and the serum iron binding capacity and serum protein were reduced by approximately 20%. This reduction was attributed to an effect of gossypol on protein synthesis. Skutches et al. (1973) gave an intravenous injection of 10 mg gossypol to pigs. This injection increased the iron concentration in the liver and bile but decreased the level of hemoglobin and hematocrit of blood. It was postulated that the gossypol reacted with iron in the liver, and the iron-gossypol complex was then excreted via the bile.

Rojas and Scott (1969) reported that gossypol caused decreased egg production, poor feed utilization, and discoloration of yolk in stored eggs. They also reported that CSM was rich in phytate which might form insoluble complexes with zinc and certain proteins.

Gossypol has a high affinity for iron (Skutches et al., 1973). This property is utilized to reduce gossypol toxicity. Clawson et al. (1975) reported that rats fed diets containing unextracted cottonseed meal treated with ferrous sulfate solution (800 ppm of iron) and supplemented with 0.2% lysine gained weight at a rate approximately equal to that of rats fed corn-soybean meal diets.

Most gossypol is removed during oil extraction. Morgan and Willimon (1954) reported that degossypolized, solvent-extracted CSM fed to broilers as the sole source of protein was about equal to soybean meal in supporting weight gain. The diet contained 2% fish meal as a source of supplemental lysine. On the contrary, the screwpress and hydraulic CSM used alone as the main source of protein produced weight gains which were significantly less than those on soybean meal. This was because screwpress and hydraulic CSM were higher in total gossypol and oil than solvent-extracted CSM (Morgan and Willimon, 1954).

Various Methods of Fermentation

The methods used for biomodification of substrates through fungal fermentation are based on the principles that the fungi employed in fermentation are saprophytes and they require a combination of moisture, temperature, air, suitable pH and darkness for desirable activity. A successful method of fermentation depends on an optimum combination of these environmental conditions for the fungi. The environmental conditions are controlled to insure the growth of the desired species and discourage the growth of all other species (Pederson, 1971).

Although different methods of fermentation describe how various factors are combined and controlled, a brief description of some of the factors employed is appropriate.

Water: Microorganisms cannot grow in absence of water. Fungi absorb nutrients in liquid form through their cell wall and discard their waste materials in liquid form. In solid state fermentation, the substrate should be moist enough for mold growth but not wet enough to promote bacterial growth (Hesseltine, 1965). Yeast and bacteria require more moisture than filamentous fungi. Moisture requirements are affected by nutrients, temperature, oxygen and other factors (Troller and Christian, 1978). Hence, at higher moisture contents, 5% $(NH_4)_2SO_4$ in the medium supported better fermentation than 1 to 3% $(NH_4)_2SO_4$.

Temperature: Mold growth is an exothermic process (Trevelyan, 1974). Consequently, the control of temperature is very important in fungal fermentation. High temperatures accompanying fermentation inhibited mold growth at 40°C and caused reduced enzyme production (Hesseltine, 1965). Most food fermenting organisms (mesophiles) grow at temperatures between 20 and 40°C. Various methods of fermentation employed temperatures within this wide range.

Air: The growth of fungi is an aerobic process (Trevelyan, 1974). Oxygen is required for the oxidation processes to release energy. Fungi obtain their nutrients from the medium on which they grow. Filamentous fungi provide various enzymes which alter organic components of foods to provide nutrients for their growth (Pederson, 1971; Whitaker, 1978). Fermentation processes are not designed to increase the weight

of foods. In fact, there usually is a 3 to 10% loss in dry matter as a consequence of energy utilization by the fermentation agent (Whitaker, 1978; Zamora and Veum, 1979b).

There seemed to be no agreement among different reports on the optimum duration of fermentation. The duration of fermentation used by various researchers ranged from 18 hrs (Zamora and Veum, 1979b) for soybeans to 6 days for grains (Semeniuk et al., 1970). It would seem as if the duration of fermentation needed to obtain the desired product depends on the genera and species of fungi employed as well as the nature of the food being fermented. The following are various methods of solid state fermentation employed by several workers.

Fermentation of cracked soybeans was reported by Zamora and Veum (1979b). They soaked 1 kg of cracked soybeans overnight in 3 liters of water containing 7.5 ml of acetic acid. Then the beans were autoclaved at 121°C for 30 minutes, and cooled to 37°C and mixed. The inocula consisted of spores of Aspergillus oryzae or Rhizopus oligosporus that were developed on potato dextrose agar in a flask for 5 days. The inoculated beans were spread on stainless steel pans to a depth of approximately 2 cm and covered with wax paper. The beans were incubated at 37°C for 18 to 24 hrs, after which the fermented product was dried at 80°C in a hot air oven.

Hesseltine et al. (1963) described pure culture fermentation of soybeans in petri dishes. Soybeans were soaked in

water for 20 hrs at 25°C. The seed coats were removed and the seeds were boiled for 30 minutes. The water was drained and the beans were cooled. The beans were mixed with a suspension of spores of Aspergillus oryzae. The suspension was made from inoculated agar slant culture made with potato dextrose agar. The slant was incubated for 7 days. Sterile water (1.5 ml) was added to the sporulated culture to facilitate suspension. Enough inoculated beans were placed in sterile petri dishes (15 x 100 mm) until they pressed tightly against the cover. The filled petri dishes were incubated at 31°C for 20 to 32 hrs. The fermentation was judged to be completed when a compact, white cake was formed with some spore formation at the edge of the petri dish where mycelium was most in contact with air.

A cereal grain fermentation was described by Hesseltine et al. (1967). Cracked grain was soaked in water and the mixture was cooked. The excess water was drained off after cooking the residue. The residue was cooled and inoculated with viable spores of Rhizopus oligosporus NRRL2710 by adding spores suspended in water. The inoculum was grown on potato-dextrose, agar slants incubated at 27°C. The inoculated grain was lightly packed in a sterilized petri dish and incubated at 31°C for 20 to 24 hrs. Hesseltine et al. (1967) proposed a large-scale fermentation in shallow trays with perforated bottoms and covers, or fermentation in perforated plastic bags. In perforated plastic bags, mixing could be

done without much contamination.

Grain fermentation in plastic bags was described by Semeniuk et al. (1970). Grains containing 8% moisture were cracked in a roller mill in amounts of approximately 100 kg. The cracked grain was screened free of fine dust particles. About 11 to 12 lots of 3.6 kg each were placed in individual plastic bags. Each lot was wetted with one liter of tap water at 40°C and the bags were shaken several times to prevent caking. Each lot was divided into two parts and placed in paper bags which were laid flat on autoclave wire mesh rack. The mouth of each bag was folded and stapled. These were autoclaved for 30 minutes at 121°C. They were cooled and the contents of a pair of flat bags were dumped into a clear polyethylene sack which was previously rinsed with 70% ethanol and drained. The transferred grain was inoculated immediately. The inoculum was a 7- to 10-day-old sporulating culture developed on 20 to 30 grams of corresponding sterilized grain in a 300 ml Erlenmeyer flask. All cultures were established directly from spores present on agar slant cultures received from Northern Regional Research Laboratory, in Peoria, Illinois. Molding of grain in each plastic sack was promoted by laying the sack flat on a tiered shelf in a dark room at 23 to 30°C. The contents of each bag were spread uniformly and the bag was tented with a centrally placed 15 cm long sterilized wooden stick. The contents of the sack were shaken daily, or less often, depending on the apparent

progress of mold growth. After 5 to 6 days, the molded grain from each sack was spread out thinly on paper to dry for 3 to 4 days in a shaded, dry greenhouse.

Chan et al. (1975) described a method in which the source of inoculum was produced on soybeans instead of on an agar slant. Aspergilli were grown on small amounts of soybeans (25 g) in a 500-ml flask to provide molded soybeans needed for each experiment. Soybeans were cracked, water conditioned to 31% moisture, and sterilized at 120°C for 45 minutes. The beans were cooled and inoculated in plastic bags as previously described by Semeniuk et al. (1970). The inoculated beans were then incubated in a dark chamber for 2 to 3 days at 23 to 30°C. The sack was shaken twice in the interim. The fermented beans were spread to air-dry for 2 to 3 days on flat racks in a dry, greenhouse hallway. The dried, fermented beans were ground in a coffee grinder.

A method of fermenting cassava meal was described by Trevelyan (1974). Water was added to cassava meal until a moisture level of 47% was attained. The mixture was made into a dough. The dough was extruded and autoclaved. The extrusion process was done to increase the surface area and to provide for adequate aeration. Prior to fermentation, cassava meal was enriched with $MgSO_4$ (2 g), KCl (10 g), $NH_4H_2PO_4$ (20 g) and urea (20 g) per kg of cassava. Other workers have demonstrated the usefulness of inorganic nitrogen sources for fungal growth. Kobayashi et al. (1968) increased

the production of phytase by adding .5% $(NH_4)_2SO_4$ to a basal medium of rice bran being fermented by Aspergillus terrus. Hesseltine et al. (1963) reported that asparagine, $(NH_4)_2SO_4$ and urea were good sources of nitrogen for Aspergillus oryzae. Imrie (1973) found that $(NH_4)_2SO_4$ and $(NH_4)_2PO_4$ were appropriate inorganic nitrogen sources for fungal growth.

Vuori and Nassi (1977) employed a continuous culture fermentor for the unsterilized aerobic fermentation of poultry manure. The fermentor had a mixer with speed of 500 rpm and, during fermentation, air was passed in at 10 liters per minute. Fermentation temperature was kept at 28°C.

Ko and Hesseltine (1962) described a unique method of producing the Indonesian, fungus-fermented food, tempeh, from soybeans. Soybeans were soaked in water overnight, after which the seed coat was removed and the beans were cooked for 30 minutes. The beans were cooled and inoculated with tempeh from a previous batch (fresh or dried and broken pieces). The inoculated beans were packaged into 1 x 5 x 10 cm size banana leaves and kept at room temperature for 1 to 2 days. The beans were also fermented on bamboo trays. The beans were spread to a one-inch thickness on bamboo trays and covered with several layers of banana leaves. A heavy weight was placed on the leaves to press them down and prevent excess aeration.

Liquid medium fermentation was described by Smith et al.

(1975). Aspergillus oryzae was grown on 3% ground barley in 2800-liter aseptic culture with 0.4% $(NH_4)_2SO_4$ added as a nitrogen source. The pH was kept at 4.5. The mycelium was separated by filtration.

Commercial application of solid state fermentation for the production of diastase was described by Duddington (1961). About 227 kg of bran was put into a rotatory drum. It was mixed and sterilized with steam. After cooling, the bran was inoculated with spores of Aspergillus oryzae. The inoculated bran was spread evenly on trays that were loaded into a growing chamber previously sterilized with formaldehyde. The fungus was allowed to grow on the bran for 15 hrs at a temperature of 37.7°C and a relative humidity of 70%; then, the temperature was reduced to 32.2°C for another 25 hrs. The diastase was extracted with 20% alcohol and later precipitated with 70% alcohol.

Biochemical and Physical Changes Due to Fermentation

Fermentation is an enzyme induced chemical alteration in foods. The enzymes involved may be produced by microorganisms or they may be indigenous to the food (Pederson, 1971). During fermentation, the energy component of the substrate is greatly depleted for metabolism and growth of the microorganism. In the process of decomposing organic matter, some of the substances produced by microbes may be very useful to animal nutrition.

Protein and amino acids

Fungi, like other plants, synthesize proteins and may synthesize some or even all the common amino acids from carbohydrate and simple sources of nitrogen (Hall, 1962). Quinn et al. (1975) and Zamora and Veum (1979a) found that the percentages of lysine, threonine and methionine of soybeans increased slightly as a result of solid state fermentation with Aspergillus oryzae or Rhizopus oligosporus. Microbial production of amino acids has a definite advantage over chemical synthesis because the amino acids produced by the former are exclusively in the biologically active form (Kinoshita, 1959).

Anderson and Jackson (1958) compared the amino acid composition of filamentous fungi, yeasts and bacteria (Table 2). The molds reported by Anderson and Jackson (1958) contained 1.04 to 1.59% lysine and 0.22 to 0.39% methionine. The values of these amino acids in the molds were much lower in concentration than in cottonseed meal or soybean meal. Hall (1962) reported that filamentous fungi were quite variable in their content of essential amino acids and generally contained lower levels of amino acids than either bacteria or yeast. The data in Table 2 by Anderson and Jackson (1958) also revealed that A. niger was lower in protein and all the essential amino acids than yeast, bacteria and oil seed meals.

The crude protein content of soybeans fermented by Aspergillus oryzae and Rizopus oligosporus was increased by 5 percentage units (Zamora and Veum, 1979a). Quinn et al. (1975)

Table 2. Essential amino acids in microorganisms and two oil seed meals; percentage of dry weight (Anderson and Jackson, 1958)

Item	*A. niger*	Brewer's yeast	*E. coli*	SBM[a]	CSM[b]
Crude protein	32.56	46.13	75.00	45.29	41.40
Arginine	1.04	1.41	3.12	3.38	4.59
Histidine	.90	1.47	.94	1.12	1.10
Isoleucine	.80	2.86	3.21	2.49	1.33
Leucine	1.48	3.74	6.21	3.48	2.41
Lysine	1.04	3.74	2.88	2.78	1.71
Methionine	.22	1.26	1.52	.63	.52
Phenyalanine	.85	1.91	2.07	2.20	2.22
Threonine	1.11	2.77	2.29	1.82	1.32
Tryptophan	.26	.61	.52	.76	.41
Valine	1.09	2.49	4.52	2.45	1.89

[a]SBM = soybean meal.

[b]CSM = cottonseed meal.

fermented solvent defatted peanut flour (SDPF) with different types of fungi. They found that the amount of increase in crude protein due to fermentation depended on the mold employed. Quinn et al. (1975) reported that there was no actual increase in protein weight as a result of fermentation. There was a loss of nonprotein volatiles during the fermentation process, which accounted for the proportionate increase in

protein content of peanut press cake. An increase in ash content was also noted. Van Veen et al. (1968) found that, although crude protein in fermented peanut press cake increased slightly, the true protein decreased from 94% to 74% of the crude protein during fermentation.

Production of enzymes

Enzymes are proteins specialized to catalyze biological reactions. The earlier name of enzymes was "ferments", and the process of enzyme action in breaking down of sugars to acids and alcohol was termed fermentation (Lehninger, 1977). During fermentation, the growing fungi secrete several enzymes which hydrolyze various components of the food or medium. Enzymatic hydrolysis is a key biomodification in the medium which supplies the fungi with nutrients. The fungi, in turn, use the nutrients to synthesize their body components resulting in growth.

Protease Proteolysis during fermentation resulted in shortened protein chains and changed texture and flavor of high protein foods (Whitaker, 1978). Neutral and acid proteases and peptidases of Aspergillus oryzae or Aspergillus soyae hydrolyzed soybean and wheat protein culminating in the formation of peptides and free amino acids (Beuchat, 1978). Hence, there was an increase in the free amino acids and peptides in fermented soybeans, peanut cake and cottonseed flour (Beuchat, 1976; Whitaker, 1978; Plating and Cherry,

1979). Quinn and Beuchat (1975) found that the nitrogen solubility of peanut flour fermented with Mucor hiemalis was increased from less than 5% of peanut flour in the nonheated control to about 34% in the fermented flour. The nitrogen solubility was measured at a pH of 4 to 5. An increased nitrogen solubility was attributed to protein hydrolysis by fungal, acid proteases to form peptides and free amino acids. It was shown (Beuchat, 1976) that free amino acids contributed 12.7% of the amino acids of peanuts fermented with Aspergillus elegans for 98 hrs.

Carbohydrate enzymes Several workers have reported the presence of amylase in fungi which hydrolyzed starches to sugars (Christensen, 1969; Whitaker, 1978; Hesseltine, 1965). Aspergillus oryzae is used in commercial production of diastase (Duddington, 1961). van Veen et al. (1968) reported that carbohydrate contents of fermented peanuts decreased significantly when compared to unfermented peanuts.

Herr et al. (1978) reported cellulolytic activity of fungi. Aspergillus niger produced cellulase which hydrolyzed cellulose to cellobiose and glucose. About 33 mg of cellobiose and glucose were produced from 100 mg of cellulose powder after 4 days. Trichoderma lignorum produced 46 mg of cellobiose and glucose from 100 mg of cellulose powder in 2 days. More evidence of cellulotytic activity was reported by Zamora and Veum (1979b). They found that the crude fiber decreased and gross energy increased in soybeans fermented with

A. oryzae or R. oligosporus. They issued no explanation for the increase in gross energy. Murata et al. (1967) and Quinn et al. (1975) found that crude fiber and ash increased due to fermentation. The increase in crude fiber was attributed to losses of volatiles during fermentation. The volatiles were low molecular weight acids, carbon dioxide, esters, aldehydes, ketones and other aromatics evolved as by-products of fungal fermentation. van Veen et al. (1968) and Wang et al. (1968) agreed that crude fiber, ash and crude protein increased during fermentation, and that there was a decrease in total carbohydrate.

Phytase Rojas and Scott (1969) reported that A. ficcum NRRL3135 secreted the enzyme phytase. In vitro hydrolysis of 41% protein cottonseed meal with this fungal phytase improved phosphorus availability and also increased the metabolizable energy value by 32%. In addition, the phytase freed some protein from protein-phytate complexes, and apparently brought about a reduction in the gossypol toxicity of the glanded meals. Phytase hydrolysis of the phytin also produced a marked reduction in zinc requirements of chicks. Kobayashi et al. (1968) reported that phytase hydrolyzed more than 95% of the phosphate linkages of sodium phytate in rice bran. This observation was made when 35 g water was added to 100 g rice bran in solid state fermentation with A. terrus.

Lipase Percentage crude fat content of fermented products remained unchanged (Zamora and Veum, 1979b; Quinn et al., 1975; van Veen et al., 1968). However, Beuchat (1978) reported that there was lipase activity in soybeans fermented with A. oryzae. The lipase activity could cause change in composition of lipids during fermentation. Quinn et al. (1975) reported that there was an increase in the concentration of linoleic acid, palmitic acid, and stearic acid, and a decrease in oleic acid when solvent defatted peanut flour (SDPF) was fermented with A. oryzae. They also noted a substantial increase in linoleic acid in the SDPF fermented by A. oryzae. Since the initial fatty acid content of the non-fermented SDPF substrate was low, the fatty acid profiles of the fungal ferments reflected the fatty acid composition of the fungus (Quinn et al., 1975).

Vitamins Quinn et al. (1975) observed significant increases in thiamin and riboflavin in peanut flour fermented with A. oryzae, R. oligosporus, M. hiemalis and A. elegans. Niacin level also was increased in peanut flour fermented with N. sitophila, R. oligosporus and M. hiemalis but not with A. oryzae. Microorganisms, including bacteria, yeast and filamentous fungi, are sources of vitamins, antibiotics and related factors used in animal rations to improve feed efficiency and increase rate of growth (Hall, 1962). Hwa et al. (1972) reported that phycomycetes (R. oligosporus, R. oryzae) used in oriental food fermentations were capable of

synthesizing antibiotics. Antibiotics can be used to reduce infection and stimulate animal growth.

Organic acids Fungi are capable of producing more than 40 acids used in industry, food and medicine (Kavaler, 1972). Organic acids are by-products of fungal metabolism during fermentation (Quinn et al., 1975). Aspergillus niger is used in commercial production of citric acid (Christensen, 1969). The spores of the fungus are sown on the surface of a liquid nutrient medium in shallow metal or enameled pans. The liquid is made sufficiently acid to keep out most competing organisms, but it is not necessary to maintain complete sterility, as in the production of penicillin. Within a few days the mold forms a thick mat of mycelium on the surface of the liquid, and by then, it has produced much citric acid into the culture. The liquid is drained off and the citric acid is precipitated as calcium citrate. Soft drink and candy manufacturers use citric acid. In 1923, the Pfizer and Co. factory producing citric acid was called "the world's largest lemon grove". Aspergillus niger is also employed in the production of gluconic acid.

Products of fungal fermentation Fungal fermentation modifies original materials organoleptically, physically and nutritionally (Beuchat, 1978). Whitaker (1978) gave a list of reasons for fermenting foods. Foods are fermented for preservation, texture, color, flavor, aroma, solubilization, improved digestibility, nutritional improvement, less cooking

time, and removal of toxic substances. Fungi are used to process various foods for any of the above reasons. Fungal enzymes are used for tenderizing meat, processing fish and producing bakery items such as breads, crackers, sugar wafers, waffles, pancakes and pizza dough (Quinn and Beuchat, 1975). For example, fungal proteinase is used to lower the viscosity of bread dough. Other foods which are products of fungal fermentation are soy sauce, tempeh and miso from soybeans and ang-kak from rice for coloring foods (Hesseltine, 1965). Roquefort cheese, named after a town in France, is fermented for its color, flavor and texture by fungi (Christensen, 1969). Blue cheese and Camembert cheese are fungi-fermented products. In most fermented products, the mold remains as part of the basic food.

Duddington (1961) reported commercial production of diastase with A. oryzae. The diastase was used for saccharifying starch for alcoholic fermentation by yeast.

Bioassay of Fermented Products

Biological assay of fermented products offers information about the digestibility and possible toxicity of the product. It also offers information on nutrient availability or the effect of the product on growth of animals. Chemical analysis of fermented products does not give any of the above information but serves to reveal changes in nutrient profile.

Although man has utilized fungi as food for hundreds of

years, the bioassays of these products for animal feeding has been done only recently. Several fermented feeds and pure fungal hyphae have been assayed with different animal species. Imrie (1973) fed mycelium of A. niger to rats and chicks and found the mycleium to be palatable, nontoxic and of good potential as an animal feed. Semeniuk et al. (1971) found that only 164 of 392 strains of Aspergillus were toxic to chicks and mice. Some of the cultures tested improved weight gain of chicks and mice when the cultures were grown on sterile soybeans or wheat. Diener et al. (1963) fermented defatted residues of peanuts with nine species of fungi isolated from domestic peanuts. They fed the fermented products to ducklings for three days and found all groups gained weight and were normal in appearance, except the group fed A. flavus. This latter group of ducks lost weight during the experiment. These researchers concluded that the only toxin-producing fungus of the nine species evaluated was A. flavus.

Quinn et al. (1975) fed fermented solvent-defatted peanut flour to rats and found that the protein efficiency ratio (PER) of fermented peanuts was not increased over properly heat-treated, unfermented peanuts. This finding is in agreement with that of van Veen et al. (1968).

Chah et al. (1975) fermented soybeans with eleven strains of aspergilli. The fermented soybeans and unfermented soybeans were used to formulate broiler diets with varying protein levels (13%, 15%, 17% and 19%). Broilers fed diets

containing soybeans fermented by 10 of the 11 species of aspergilli gave significant improvements in weight gain and feed efficiency as compared to diets containing unfermented soybeans. The responses were more pronounced with the lowest dietary levels of proteins. The carcasses of broilers fed fermented soybeans were high in protein and ash but were lower in lipids than the carcass produced from unfermented soybeans. These researchers explained that the growth promoting effect of fermented soybeans and the leaner carcass of broilers fed the fermented soybeans were associated with a greater supply of amino acids and the possibility of additional vitamins.

Chah et al. (1976a) supplemented a control diet containing unfermented soybeans with essential amino acids to simulate the amino acid composition of diets containing the fermented soybeans. Growth and feed efficiency of chicks fed these supplemented diets were equal to those of chicks fed diets containing fermented soybeans. Chah et al. (1976a) explained that the growth stimulation observed when fermented soybeans were fed in a broiler diet was due to superior amino acid balance of fermented soybeans. The soybeans were fermented with 6 species of aspergilli.

Zamora and Veum (1979a,b) found that pigs and rats fed diets containing soybeans fermented with A. oryzae or R. oligosporus had higher average daily gain than those fed unfermented soybeans. Feed efficiency also was better on fermented soy-

beans than on unfermented soybeans.

Chah et al. (1976b) reported that Japanese quail fed fermented soybeans gained significantly more weight than those fed unfermented soybeans. The fermented soybeans also improved rate of egg production and egg size of the quail slightly but not significantly.

Smith et al. (1975) fed mycelium from A. oryzae and other filamentous fungi to rats or pigs at 10% of diet. They found that all species of fungi were deficient in sulfur amino acids for the growing rats and pigs. These researchers attempted to correct this amino acid deficiency by methionine supplementation. A diet containing A. oryzae was supplemented with L-methionine and fed to rats or pigs. Smith et al. (1975) observed that net protein utilization (NPU) of the diet was not increased by L-methionine supplementation. However, NPU was increased when a diet containing Fusarium semitetum and supplemented with L-methionine was fed to rats or pigs.

Zamora and Veum (1979a) fed diets containing soybeans which had been fermented with A. oryzae or R. oligosporus to rats. They observed a 30% improvement in average daily gain of rats when the fermented soybeans were fed in one experiment but only a 12% improvement in average daily gain was noted in a second experiment.

DEVELOPMENT OF FERMENTATION PROCEDURE

Introduction

This section is a detailed account of how a method was selected from various methods examined for the development of solid state fermentation. The methods are based on the principle that the filamentous morphology of the fungi enables them to colonize and expand across solid substrate at a much faster rate than bacteria. The filamentous habit is adapted to a warm, humid substrate with adequate air.

Submerged fermentation in liquid medium, such as employed in bacterial or yeast fermentation, was avoided because that method involved complicated equipment to stir the nutrients, aerate the medium, and warm the medium, or cool it if it gets hot. The control of pH becomes critical in submerged fermentation. Submerged fermentation is complex because a liquid medium is not a natural habitat of the filamentous fungi. Hence, attention is focused on the development of solid state fermentation which requires less complicated equipment.

Aspergillus oryzae NRRL506 and Aspergillus janus NRRL1935 were the two fungal specimens used. They were obtained from Dr. C. W. Hesseltine at Northern Regional Laboratory, USDA, ARS, Peoria, Illinois.

Materials and Methods

Choice of nutrient medium for production of spores

A suitable nutrient agar medium determines the degree of fungal growth and amount of spores produced. Four nutrient media were examined in the selection of a medium which supported excellent growth and sporulation. The nutrient media were malt agar, sabaraud dextrose agar, Czapek Dox agar, and malt extract agar. These were commercial preparations in powdered form. Their ingredient composition per liter of distilled water was as follows:

Czapek Dox agar: 50 g/liter	
Sucrose	30.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	.5 g
KCl	.5 g
$FeSO_4 \cdot 7H_2O$	.01 g
Agar	15.0 g
Final pH	7.3

Malt extract agar: 33.5 g/liter	
Maltose	12.75 g
Dextrin	2.75 g
Glycerin	2.35 g
Peptone	.78 g
Agar	15.0 g
Final pH	4.6

Malt agar:45 g/liter

Malt	30 g
Agar	15 g
Final pH	5.5

Sabaraud dextrose agar:65 g/liter

Dextrose	40 g
Polypeptone	10 g
Agar	15 g
Final pH	5.7

Agar is a component that solidifies the medium. Each nutrient medium is recommended for the cultivation of fungi.

Each nutrient medium was weighed and suspended in one liter of distilled water. The suspension was heated with agitation for about a minute to melt the agar, then it was poured into test tubes to one-third capacity. The test tubes had their covers loosely fitted and the whole assembly was autoclaved at 121°C and 1.055 kg/sq cm pressure for 10 minutes. The test tubes were inclined at a 30 to 40° angle to cool and to allow the hot liquid nutrient medium to congeal as a slant. Each agar slant was inoculated with spores of A. oryzae or A. janus and incubated at 30°C for 7 days. The test tubes were examined for good growth and sporulation. The information presented in Table 3 describes the degree of growth and sporulation attained by each fungus on each nutrient medium. Growth and sporulation were evaluated as fair, moderate, good or excellent with fair as the lowest value and excellent as the highest value.

Table 3. Bases for selection of nutrient medium for spore production

Medium	Fungus	Growth and sporulation
Czapek Dox agar	A. oryzae	Excellent growth and excellent sporulation
	A. janus	Excellent growth and excellent sporulation
Malt agar	A. oryzae	Good growth but no sporulation
	A. janus	Fair growth and fair sporulation
Malt extract agar	A. oryzae	Good growth, no sporulation
	A. janus	Moderate growth, moderate sporulation
Sabaraud dextrose agar	A. oryzae	Good growth, no sporulation
	A. janus	Fair growth, fair sporulation

Czapek Dox agar was selected to be the medium suitable for excellent hyphal growth and excellent sporulation. Aspergillus janus had grayish black mycelium with a rust-like appearance. The spores were black and their production was copious. Aspergillus oryzae had white, luxuriant hyphal growth with gray spores. Some of the spores harvested from the two strains of Aspergillus were lyophilized and stored in a cool place for future use. The spores designated for

immediate use were stored in a refrigerator.

Production of spores for inoculation

Slant cultures This step insures that spores are pure stock, virile and produced in a sterile condition. Czapek Dox agar slant (Figure 1) was made as previously described. The sterile medium was inoculated in a sterile room. Air coming into the room was filtered and ultraviolet light kept the room sterile when it was not in use. The slant culture was incubated for 7 days. The spores produced were kept in a cool place ready for use in petri dish culture.

Petri dish culture This step is for the multiplication of fungal spores because the petri dish has a larger surface area than the test tube slant culture (Figure 1). Czapek Dox (35 g) and agar (15 g) were weighed and suspended in a liter of distilled water in a 2 liter Erlenmeyer flask. The suspension was heated with agitation for a minute to melt the agar. The flask was covered with aluminum foil and autoclaved at 121^{o}C and 1.055 kg/sq cm for 10 minutes. The hot liquid nutrient medium was poured into sterile plastic petri dishes which were then covered and allowed to cool. The Czapek Dox agar was dispensed in a nonsterile laboratory atmosphere where air draft was minimum. In late fall and winter, contamination of the agar was minimal but in summer the level of contamination was high. In summer, the agar was poured in the atmosphere of a sterile room. The petri dish

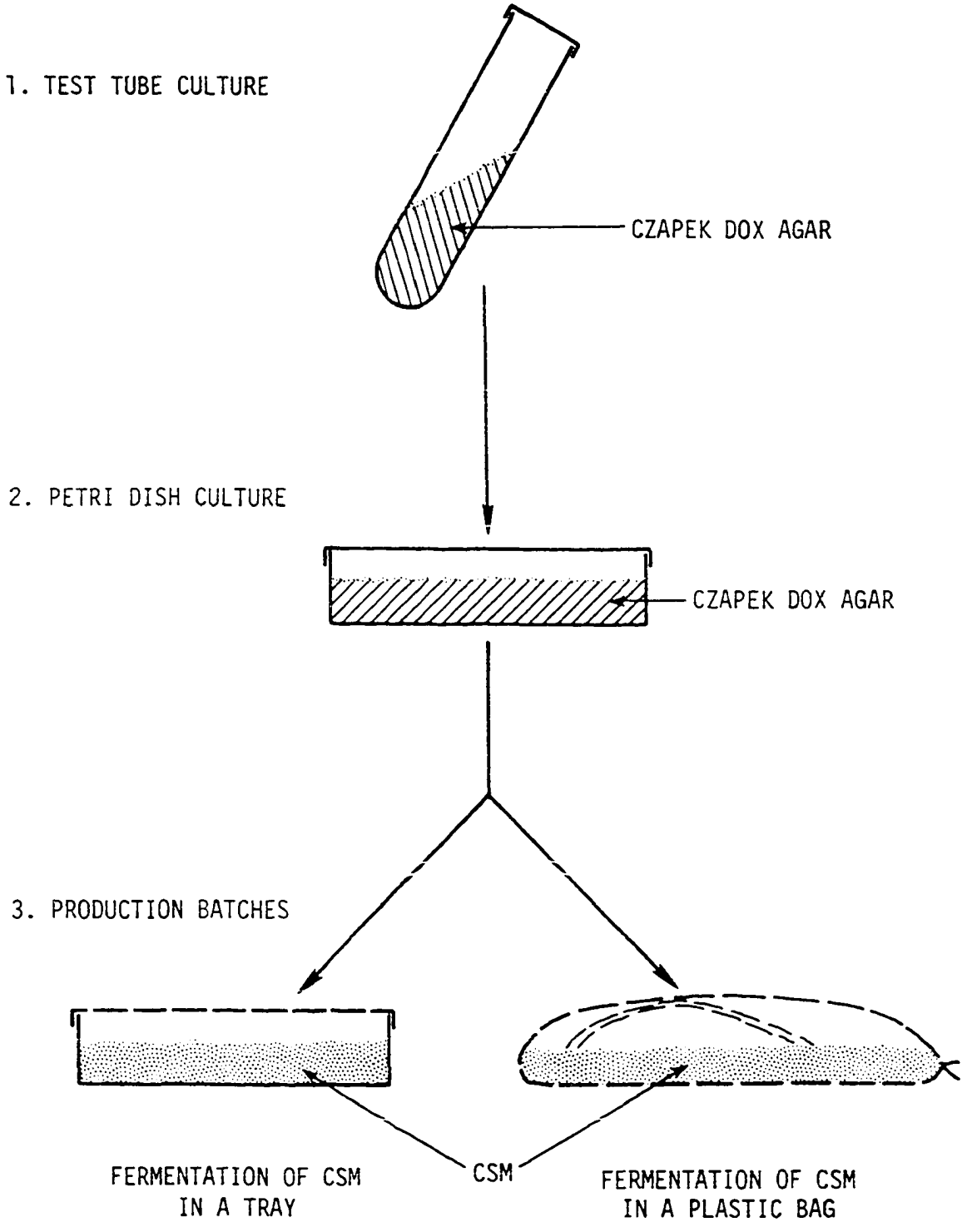

Figure 1. Stages in FCM fermentation

medium was inoculated in a sterile room with spores of the two strains of Aspergillus from the test tube cultures. One to 2 ml of sterile water were shaken in test tube slants to suspend the spores used for the inoculation. The inoculated petri dish cultures were incubated at 30°C for 7 days to facilitate production of viable spores.

An attempt also was made to produce spores on sterile moisture conditioned cottonseed meal in petri dishes. A spore suspension from the test tube cultures was used as inoculum. The inoculated cottonseed meal was incubated at 30°C. Fungal growth was rapid and spores were produced much earlier in cottonseed meal culture within 4 days than in Czapek Dox culture. The problem with using cottonseed meal culture for spore production was associated with the dark color of sterilized cottonseed meal which made it difficult to identify any possible contamination in the medium. It was much more difficult to spot contamination in cottonseed meal cultured with A. janus than with A. oryzae because A. janus had dark brown hyphae and spores.

Fermentation of cottonseed meal (CSM)

Three types of containers were investigated for use in fermentation. The containers were petri dishes, trays and plastic bags (Figure 1). All the CSM used in each container was treated alike. One hundred grams of CSM and 50 g of acidified tap water, or multiples of these, were mixed in a

Kitchen-Aid type mixer to give 33% added water in the mixture. Sulfuric acid was used to lower the pH of the tap water to 3.5. A small amount of water was used to suspend fungal spores in petri dishes. The petri dish suspension was poured into a beaker. Acidified water was added to bring the volume to the level required for a weighed amount of CSM. One to two petri dish cultures were used per 454 g of CSM, depending on abundance of spores. The inoculation and subsequent fermentation were nonaseptic. The inoculated CSM was either fermented in 15 x 100 mm plastic petri dishes, glass or metal trays or perforated plastic bags. The inoculated CSM in each container was incubated for 48 hours. The resulting fermented cake was dried at 80°C in a hot-air oven for 24 hours.

At the beginning of the experiment, the CSM used for fermentation was sterilized. CSM had water mixed with it to achieve a final water content of 33%. The mixture was placed in trays and covered with aluminum foil. This assembly was autoclaved for 10 minutes at 121°C and 1.055 kg/sq cm. The trays were cooled with the cover in place. The CSM was inoculated in a sterile atmosphere with spores from petri dish cultures. A small amount of sterile water was used to suspend fungal spores in a petri dish culture. The suspension was poured on the cooled CSM in a tray and a spatula was used to mix the spores with the CSM. The inoculated CSM was incubated in the same trays in a walk-in incubator in which the air was

not sterile.

The following are the results and discussion of the use of each type of container. The discussion will focus on the prospects and problems associated with each type of container.

Results and Discussion

Fermentation in petri dishes

The inoculated CSM was lightly packed in sterile plastic petri dishes so that 2 to 3 mm space was left at the top. These were incubated at 30°C for 48 hrs. After 48 hrs, some moisture which had condensed on the inside of the cover during early part of incubation was virtually disappeared. This indicated that the moisture level of the culture was decreasing. After 48 hrs, the mycelium had formed an intricate network that penetrated the entire CSM. The fermented CSM was taken out as one piece of white cake when A. oryzae was the fermenting organism; A. janus formed a grayish, fragile cake. Forty-eight hours seemed to be adequate time for fermentation because the top of the culture was dry thereafter. Kihlberg (1972) reported that filamentous fungi can double their masses in 4 to 12 hrs during fermentation. Also, it was found that percentage protein of the fermented CSM remained almost constant after 48 hrs of fermentation. Loss of moisture through the edge of the petri dish probably prevented further development. A petri dish is suitable for solid state fermentation because the shape is suitable for adequate gaseous exchange,

elimination of excess heat of fermentation and loss of moisture which otherwise could drip into the culture from underside of the cover. However, petri dishes are small and not suitable for large scale fermentation. The moisture loss during fermentation could not be controlled.

Fermentation in trays

The inoculated CSM was placed in glass and metal trays and spread to a depth of 1 to 2 cm. Some of the trays were covered with perforated aluminum foil (Figure 1) and others were covered with nonperforated aluminum foil to retain the microhumidity of the culture. The holes were 1 to 2 mm in diameter and were made with a pencil tip. The trays were not perforated. Those trays covered with nonperforated aluminum foil were not air-tight and gaseous exchange occurred around the edge of the trays. Trays containing sterilized inoculated CSM were all covered with perforated and nonperforated aluminum foil. The trays were placed on racks in a growing chamber kept at 30°C. The progress of the fermentation was observed for 7 days.

By 48 hrs, fungal growth was complete throughout the CSM. The aroma of fermentation was present. Moisture had accumulated on the inside of the nonperforated aluminum foil and was dripping into the culture. The humidity was high and the temperature of the culture was about 38°C. The dripping water caused putrifaction and copious release of ammonia.

This trend was observed in sterilized and nonsterilized CSM. This observation agrees with those of Young and Wood (1974) that moisture and lack of oxygen facilitated the development of bacteria. A solution to this problem was attempted. A paper towel was taped on the inside of the nonperforated aluminum foil cover to absorb the moisture. The positive effect of this was short-lived. The paper became too wet to hold any more water and the water-soaked paper peeled off. The CSM at the edge of the tray which had most contact with air was well-fermented. The central part of the tray had some areas not well-colonized by the fungus.

The problems with fermentation in trays covered with aluminum foil included control of excess water, high temperature and inadequate aeration. These problems also were observed with sterilized CSM fermented in trays covered with aluminum foil and which had adequate water added during aseptic inoculation. The process of sterilizing CSM evaporated much water. Hence, the surface of the substrate, which had little water added during inoculation, became dry during fermentation. There was not enough humidity to support growth on the surface although there was some growth below. Because the air in the growing chamber was not sterile, the fermenting culture did not remain sterile and some fungal contaminants were noticed.

Fermentation in trays covered with perforated aluminum foil had inadequate humidity to initiate fungal germination

at the top of the culture. However, fungal growth occurred in the interior of the culture where moisture was adequate. Lack of surface moisture was prevented by initially covering the perforated aluminum foil with a nonperforated plastic sheet for 24 hrs. After 24 hrs, the plastic sheet was removed because good fungal growth had occurred and moisture droplets had formed on the underside of the aluminum foil. Excess moisture and heat of fermentation then escaped through the perforations. Adequate ventilation also occurred through the perforations to produce a more uniform fermentation. After 48 hrs, the top of the culture became quite dry.

Fermentation in trays covered with perforated aluminum foil is a suitable method because the CSM was uniformly fermented if the layer of CSM was within 1 to 2 cm thick. The only constraint to prolonged fermentation, as in petri dish, was the uncontrolled loss of moisture. The trays used here were small (46 x 30 cm). Probably, large wooden trays with perforated bottoms could provide enough area suitable for large-scale fermentation.

Fermentation in perforated plastic bags

The inoculated CSM was spread in transparent, perforated plastic bags (51 x 58 cm; 58 x 122 cm) to a depth of approximately 1 to 2 cm. Holes were made in the bags with cut edges of wire mesh. The diameter of the wires were 1 mm and 2 mm. Since no piece of the bag was removed to make a clear-

cut hole, the holes made in the bag resembled partially closed valves. Hence, the inoculated CSM did not leak out of the bag. The bags were tented with strips of wire mesh to facilitate aeration, and the mouths of the bags were tied, taped or clipped to retain the microhumidity. The height of the tent was 8 to 12 cm above the inoculated CSM. The bags containing the inoculated CSM were incubated in a dark, aerated chamber for 48 hrs. Turning was not required because the bags were perforated on both sides and placed on a wire rack for adequate aeration. At 48 hrs of incubation, the resulting cake was dried at 80°C in a hot-air oven for 24 hrs. The dried product was pulverized and used as a component of the experimental diets.

During fermentation, some moisture accumulated on the inside of the plastic bag and partially occluded the holes in the bag. The temperature of the culture rose to 38°C. Moisture and excess temperature were controlled as follows. At the time the plastic bags were set on the racks in the growing chamber, 2 to 3 openings (5 x 3 cm) were made at intervals of about 15 to 20 cm along the mid-top of the bag. These openings were closed with a sticky tape. After 24 hrs, fungal growth had been established and some moisture had accumulated on the inside of the plastic bags. At this time, some or all the tape was removed from the openings depending on the degree of moisture accumulated. The tape was peeled slowly to prevent moisture droplets from falling into

the culture. Opening the bags also prevented an undesirable temperature build-up and provided extra ventilation needed at that very active period of fermentation.

It was also found that excess height of the plastic tent caused excess air circulation above the culture and dried the surface. A height of 8 to 10 cm was adequate and was just enough for the hand movement to spread the inoculated CSM and install the wire supports.

It was observed that the initiation of germination of fungal spores was delayed when thick plastics were used instead of thin plastics. Probably, the thick bag had a higher insulation value than thin bags, and slowed the transfer of heat from the growing chamber into the bag.

General Comments on Methods

The general purpose of media sterilization is to prevent contamination of the end product and to insure that all the nutrients are available for growth of the desired organism. Absolute or virtual sterility is easily obtained on a small scale in the laboratory by autoclaving for short periods. On an industrial scale, however, difficulties may arise because of the large quantities of fermentable material involved. A solution may be to produce large quantities of spores in a sterile condition, and then use them to mass inoculate an acidified, production batch. Hopefully, the massive inoculum would outgrow any contaminants present (Riviere, 1977). This

principle has been employed in the production of unsterilized fermented CSM in this experiment.

The problems of solid state fermentation include the maintenance of a constant moisture level, desirable temperature and adequate aeration. Also, solid state fermentation occupies a relatively large amount of space because the substrate must be spread out thinly enough for adequate aeration and heat loss. Generally in western countries, solid state fermentation is avoided because of the space it occupies and the harvesting difficulties (Riviere, 1977).

Plastic bags are cheap and easily modified to control aeration, temperature and moisture for a limited time. Later in the experiment when the procedures were established, opaque or translucent trash bags (58 x 122 cm) were used with very good results. Maintaining an adequate moisture level for an extended period of fermentation was not achieved with any of the containers used. The evaporated moisture was never replenished and fungal growth was halted when the moisture level became low. However, adequate fermentation was obtained for 48 hrs. Walter and Mhatre (1965) reported that the culture could be prevented from drying by maintaining a high relative humidity in the growing chamber. The authors suggested that steam could be used to maintain the humidity of a growing chamber.

Moisture conditioned CSM is not as granular as moisture conditioned cracked grain or soybeans. High moisture level

in CSM occludes air circulation during fermentation and induces the development of bacteria (Young and Wood, 1974). Semi-solid substrates require mechanical devices to aerate the media and to remove excess heat of fermentation as experienced with yeast and bacteria during fermentation in liquids.

Conclusion

Of the various containers explored for small-batch fermentation, perforated plastic bags were selected for use because they were cheap and easy to use. With minor modification, plastic bags provided adequate aeration with accompanying temperature control. Humidity of the culture could be controlled much longer than in the petri dish or tray methods. Also, plastic bags provided a relatively large surface area for fermentation.

FUNGAL-FERMENTED COTTONSEED MEAL; EFFECT ON GROWTH OF BROILERS

Introduction

Fermentation processes long have been associated with bacteria or fungal biomodification of complex organic substances into more useful products. For centuries, oriental cultures have employed filamentous fungi to transform soybeans into tempeh, a product with a more desirable flavor, aroma and texture, and a higher digestibility for humans than cooked soybeans (Hesseltine, 1965). Traditionally cooked soybeans have been unpopular as food since they do not soften during cooking and are relatively difficult to digest.

Protein quality, rather than quantity, has been the concern of monogastric nutritionists even before the advent of the amino acid analyzer. It is not surprising that there is the current surge of interest among many investigators in the use of fermentation with specific filamentous fungi to improve the nutritional quality of oil seeds and oil seed meals. Generally, vegetable proteins are deficient in one or more essential amino acids. Evidence from Chah et al. (1976a) and Zamora and Veum (1979b) indicated that broilers and pigs fed diets containing soybeans fermented with A. oryzae gained significantly more weight and converted feed to gain more efficiently than those fed diets containing unfermented soybeans. This growth stimulation was attributed to increased

protein content and superior amino acid balance of fermented soybeans. Beuchat (1976) reported an increase in free amino acid content of fermented peanuts over raw peanuts.

Imrie (1973) fed mycelium from A. niger to rats and observed that the mycelium was palatable and nontoxic for growing rats and had good potential for use in animal feed. Smith et al. (1975) fed 10% pure mycelium of A. oryzae to rats and pigs and found that all species of fungi were deficient in sulfur amino acids.

In addition to nutrient profile changes due to fermentation of oil seed materials, other biomodifications occur that may improve digestibility and nutrient availability to animals. Proteolytic activity of fungal enzymes caused major changes in texture and flavor of high protein foods (Whitaker, 1978). The fungal enzymes digested plant constituents and presumably made these materials more digestible by animals (Beuchat, 1978). Aspergillus oryzae produced strong proteolytic enzymes in peanut seeds in a process of fermentation (Cherry et al., 1976). Rojas and Scott (1969) hydrolyzed cottonseed meal (CSM) in vitro with phytase extracted from A. ficum NRRL3135. Hydrolysis improved phosphorus availability from CSM and increased the metabolizable energy value of CSM by 32%. Also, the phytase liberated protein from protein-phytate complexes and apparently caused a reduction in gossypol toxicity of the glanded CSM.

Cottonseed meal contains low levels of lysine and

methionine (Smith, 1970). Also, it contains 2 to 3 times as much fiber as 44% protein soybean meal (Naber and Morgan, 1957). Rojas and Scott (1969) reported CSM had a high phytate content which formed undesirable complexes with zinc and proteins. They also reported the presence of gossypol which caused poor feed utilization and decreased egg production.

Cottonseed meal has received little attention as a fermentable substrate although it has poor protein quality and low metabolizable energy.

The experiments described here were designed to determine the effect of fungal fermentation of CSM on the quality of proteins of the meal and on growth of broilers fed fermented CSM.

Materials and Methods

Solvent extracted CSM (41% protein, .04% free gossypol) was used as substrate for fungal fermentation by A. oryzae NRRL506 and A. janus NRRL1935. The fungal strains used in this study were obtained from Dr. C. W. Hesseltine at Northern Regional Laboratory (USDA, ARS, Peoria, Illinois). The inocula were spores grown on a nutrient medium composed of Czapek Dox broth (3.5 g/100 ml of water) and agar (1.5 g/100 ml of water). The agar was added to solidify the medium. The fungi were grown on the slant from this nutrient medium for 7 days at 30°C and in the dark to obtain viable spores. Subsequently, larger batches of the spores were produced in

15 x 100 mm petri dishes for another 7 days. The spores were suspended in acidified distilled water (pH 3.5) and the volume was adjusted until water constituted 33% of the total mixture. In more specific terms, 50 g of water was added to 100 g of CSM. The fungal suspension was mixed with a weighed amount of CSM in a Kitchen-Aid type mixer.

The inoculated CSM was spread in perforated plastic bags to a depth of approximately 1 to 2 cm. The bags were tented with strips of wire mesh to facilitate aeration, and the mouths of the bags were tied, taped or clipped to retain microhumidity. The height of the plastic tent was 7-13 cm above the inoculated CSM. The bags containing inoculated CSM were incubated at 30°C in a dark, aerated chamber for 48 hrs. Turning was not required because the bags were perforated on both sides and placed on a wire rack for adequate aeration. After 48 hrs of incubation, the resulting cake was dried at 80°C in a hot-air oven. It was pulverized and used as a component of the experimental diets. Nitrogen was analyzed by the Kjeldahl method (Association of Official Agricultural Chemists, 1975). Amino acids were determined according to Moore (1963) on a Durrum Autoanalyzer Model D-400.

Four experiments were conducted. Experimental diets for all three experiments are shown in Table 4. All CSM and fermented CSM (FCSM) diets were supplemented with 5% fat. All diets were isocaloric (2940 kcal ME/kg) and isonitrogenous

Table 4. Composition of control and fermented cottonseed meal (FCSM) diets[a]

Ingredient	SBM	CSM[c] +L+M	CSM	FCSM[b] A. oryzae NRRL506	FCSM A. janus NRRL1935
	----------	----------	-%	----------	----------
Yellow corn (8.2% prot.)	65.51	54.30	54.30	57.66	57.66
Soybean meal (44% prot.)	24.49	-	-	-	-
Cottonseed meal (41% prot.)	-	30.70	30.70	-	-
FCSM (46% prot.)	-	-	-	27.34	27.34
Blood meal (80% prot.)	3.00	3.00	3.00	3.00	3.00
Alfalfa meal (17% prot.)	2.00	2.00	2.00	2.00	2.00
Limestone	1.50	1.50	1.50	1.50	1.50
Dicalcium phosphate	2.50	2.50	2.50	2.50	2.50
Vitamin premix[d]	0.50	0.50	0.50	0.50	0.50
Salt TM premix[e]	0.50	0.50	0.50	0.50	0.50
Fat	-	5.00	5.00	5.00	5.00

[a]Composition of diets were the same for all experiments but fermented CSM was supplemented with lysine and methionine in experiment 3. In experiment 1, sterilized CSM was fermented. In experiment 2, unsterilized CSM was fermented.

[b]FCSM = fermented cottonseed meal.

[c]L = L-lysine (0.41%), M = D·L-methionine (0.25%).

[d]0.50% vitamin premix supplied the following per kg: 750 IU vit. A, 1500 IU vit. D_3, 5 IU vit. E, 20 µg vit. B_{12}, 1 mg vit. K, 6 mg Rib., 22 mg CaPA, 75 mg niacin, 400 mg choline chloride.

[e]Salt-trace mineral premix: 70 ppm Mn, 40 ppm Zn, 20 ppm Fe, 6 ppm Cu.

(20% protein). There were two positive control diets and one negative control diet for all the experiments. Diets containing soybean meal (SBM) and CSM supplemented with lysine and methionine served as positive control diets. The diet containing CSM and not supplemented with amino acids served as the negative control.

Experiments 1 and 2 differed in their pretreatment of CSM before fermentation. In experiment 1, CSM was sterilized, cooled and inoculated with fungal spores. In the second experiment, CSM was not sterilized, but it was acidified to suppress bacterial activity. Experiment 3 was similar to experiment 2 except that the fermented CSM diets were supplemented with lysine and methionine at levels corresponding to 50% of the levels of each of these amino acids included in CSM positive control diet.

In experiment 4, CSM was enriched with 1, 2, 3, 4, and 5% ammonium sulfate (AS) prior to fermentation. Equal amounts of enriched FCSM and nonenriched FCSM were used to formulate broiler diets. The diets containing enriched FCSM were compared with diets containing nonenriched FCSM and unfermented CSM. The positive control diets were a SBM diet and a CSM diet that was supplemented with one-half of lysine and methionine needed to meet NRC (1977) requirements.

The broiler chicks were fed the corn-soybean control diet during the adjustment period, 1 to 7 days of age. The chicks then were selected and classified into weight range

groups. Replicates of chicks were selected by taking one or more chicks from each weight group.

Triplicate pens of male broiler chicks were fed the experimental diets from 7 to 19 days of age in experiment 1, 7 to 21 days in experiment 2, and 7 to 28 days in experiments 3 and 4. Each experiment was of a completely randomized design utilizing 5 broilers per replicate and 3 replicates per treatment. Analysis of variance according to Snedecor and Cochran (1967) was used to partition sources of variation. Duncan's (1955) multiple range test was used to compare treatment means.

EVALUATION OF FERMENTED PRODUCTS

Experiment 1. The Nutritive Value of Sterilized Fermented Cottonseed Meal in Broiler Diets

Results

The cottonseed meal (CSM) tested in this experiment was sterilized prior to fermentation with Aspergillus oryzae and Aspergillus janus. Chemical and biological assays were used to evaluate the product of fungal-fermented cottonseed meals (FCSM). Chemical analysis was complemented by broiler chick assay because chemical analysis was only an indication of nutrient composition. Broiler chick assay indicated how effectively the product was digested and the nutrients were used by chicks.

Chemical assay Chemical analysis showed that fermentation caused some changes in the nutrient profile of CSM (Table 5). Fermentation increased crude protein by 5 percentage units and acid detergent fiber (ADF) by 3.5 percentage units. There was no significant change in crude fat but gross energy of FCSM decreased by 2.5%.

Amino acid analysis (Table 5) indicated that fermentation significantly ($P \leq .05$) increased the lysine (27 to 38%), methionine (10%), and branched chain amino acid contents of the protein as compared with nonfermented CSM. The analysis also showed that the concentrations of most essential amino acids were increased by fermentation. There was a 2.8

Table 5. Essential amino acid, crude fat, ADF, crude protein and GE[a] content of CSM and fermented CSM (percent of dry matter), experiment 1

Item	Non-fermented CSM	*A. oryzae* sterilized fermented CSM	*A. janus* sterilized fermented CSM
Crude fat	5.47	4.2	4.4
ADF	15.37	18.81	18.85
GE	4647	4508	4569
Crude protein	41	46.27	46.0
Arginine	3.76	3.73	3.82
Histidine	1.02	1.45	1.24
Isoleucine	1.20	1.70	1.48
Leucine	2.02	2.34	2.52
Lysine	1.46	1.84	1.89
Methionine	.57	.63	.63
Phenylalanine	1.92	2.16	2.17
Threonine	1.12	1.29	1.41
Tryptophane[b]	-	-	-
Valine	1.52	1.83	1.96
% α-AA[c] of meal	31.64	34.41	35.80

[a]GE = gross energy in calories per gram.

[b]Analytical method employed oxidized tryptophane.

[c]AA = amino acid.

percentage unit increase in total α-amino acid in CSM fermented by A. oryzae and a 5 percentage unit increase in total α-amino acid in CSM fermented by A. janus.

The FCSM contributed more lysine, methionine and isoleucine to the diets (Table 6) than did the unfermented CSM although, based on the protein content of the fermented product, the amount of FCSM included in the diet was lower than the amount of unfermented CSM used in the negative and positive control diets. For example, diets containing FCSM had lysine levels of 0.88 to 0.92% as compared with 0.82% lysine in diets containing unfermented CSM. The diet containing soybean meal and the positive control CSM diet contained 1.26 and 1.28% lysine, respectively. Also, isoleucine levels were 0.69 to 0.72% in diets containing FCSM as compared with 0.65% isoleucine in the negative CSM and positive CSM diets. But the diet containing soybean meal had 0.92% isoleucine. Methionine level was 0.21% in diets containing FCSM as compared with 0.29% methionine in CSM diet. The diet containing soybean meal and the positive CSM diet contained 0.53 and 0.43% methionine, respectively.

Biological evaluation The average body weight gain, feed conversion, and feed consumption data are summarized in Table 7. Weight gains of chicks fed diets containing FSCM obtained from fermentation with either A. oryzae or A. janus were significantly ($P \leq .05$) greater than those of chicks fed the unsupplemented CSM diet. The chicks fed FCSM diets gained

Table 6. Essential amino acid composition of diets (percent of diet), experiment 1

Amino acids	SBM	CSM +L+M[a]	CSM	FCSM *A. janus* sterilized	FCSM *A. oryzae* sterilized
Arginine	1.19	1.80	1.80	1.43	1.52
Histidine	.51	.55	.55	.54	.58
Isoleucine	.92	.65	.65	.72	.69
Leucine	1.89	1.67	1.67	1.61	1.70
Lysine	1.26	1.28	.82	.81	.92
Methionine	.53	.54	.29	.31	.31
Phenylalanine	1.22	1.12	1.03	1.05	1.07
Threonine	.78	.73	.71	.68	.78
Tryptophane	.24	.23	_[b]	_[b]	_[b]
Valine	1.07	1.09	1.09	1.03	1.12

[a]M = D·L-methionine (0.25%), L = L-lysine (0.41%).

[b]Analytical method employed oxidized tryptophane.

28 to 31% more weight than did the chicks fed the CSM treated group. The potential of the FCSM becomes obvious when it is compared to the CSM diet which was adequately supplemented with lysine and methionine. Broiler chicks fed the supplemented CSM diet gained 29% more weight than chicks fed diets containing FCSM. Also, broiler chicks fed the supplemented CSM diet gained 66% more weight than those chicks fed

Table 7. Effect of feeding sterilized fermented cottonseed meal on weight gain and feed efficiency of broiler chicks (7-19 days of age)[a]

Diets	Weight gain[b] (g)	% change over CSM[c]	Feed/ gain (g)	Feed consumed/ chick
SBM	220.0 a	+92	1.73 d	380 a
CSM+L+M	190.3 b	+66	2.00 c	381 a
CSM	114.3 d	-	2.62 a	299 c
FCSM, A. oryzae	146.5 c	+28	2.38 b	348 b
FCSM, A. janus	149.3 c	+31	2.24 b	334 b

[a]Means in the same column and with different letters are significantly different ($P \leq .05$).

[b]Mean weight gain per chick.

[c]Percentage increase in weight gain as compared to cottonseed meal (CSM) diet.

unsupplemented CSM diet.

Mean weight gain by chicks fed diets containing FCSM produced with A. oryzae did not differ significantly ($P \geq .05$) from the weight gain by chicks fed diets containing FCSM produced with A. janus.

The substitution of CSM for soybean meal (SBM) in diets resulted in a significant depression of weight gain by broiler chicks (Table 7). Supplementing the CSM diet with lysine and methionine improved chick weight gain significantly as compared with CSM diet alone. But the supplemented CSM diet

failed to support a rate of gain comparable to that of chicks fed the SBM diet.

Feed efficiency Feed efficiency was significantly ($P \leq .05$) improved for chicks fed diets containing FCSM as compared to chicks fed the unsupplemented CSM diet. In this experiment, feed efficiency was not significantly different between chicks fed diets containing the product of A. oryzae fermentation and the product of A. janus fermentation. Feed efficiency of broiler chicks was depressed when CSM was substituted for SBM in diets. Feed efficiency of broilers was improved significantly ($P \leq .05$) when the CSM diet was supplemented with lysine and methionine. The feed efficiency of chicks fed the amino acid supplemented CSM diet was significantly improved as compared to that of chicks fed FCSM diet, but was poorer than obtained on SBM diet.

Feed consumption There was no significant difference between the amounts of feed consumed by chicks fed the SBM and chicks fed supplemented CSM diets. Also, the amounts of feed consumed by chicks fed diets containing A. oryzae or A. janus fermentation products did not differ significantly. Chicks fed the CSM diet consumed significantly less feed than chicks fed any other diet.

Mortality There was no mortality in any of the treatment groups during the 19-day experiment.

Discussion

A new protein source can be evaluated in two different ways. A new protein ingredient could be substituted for SBM in other protein sources of known quality. Also, it could be used at a level which furnishes adequate amounts of essential amino acids in the ration. In this experiment, FCSM was considered a new protein source and it was used to replace SBM in the diet.

The feeding trial showed that broiler chicks fed diets containing FCSM grew faster and utilized feed more efficiently than those fed diets containing unfermented CSM. The improved growth response and feed conversion seemed to be due to an increased α-amino acid content of FCSM. A portion of the improvement also could have resulted from a favorable change in essential amino acid profile as a result of synthesis of fungal proteins. For example, the levels of lysine, methionine and isoleucine increased in FCSM and diets containing FCSM as compared to the levels in CSM and CSM diets, respectively (Tables 5 and 6). Imrie (1973) reported that fungal protein had a high digestibility, and the lysine of the protein was 95 to 100% available when measured with chick assay. In the research reported here, the chicks fed diets containing FCSM gained an average of 29% more weight than chicks fed diets containing unfermented CSM. This finding is in agreement with those of Zamora and Veum (1979a) who observed a 30% increase in weight gain of rats fed diets

containing 20 to 26% fermented soybeans. Also, Chah et al. (1976a) observed a 7 to 12% increase in weight gain of broilers fed diets containing soybeans fermented by any of the six species of Aspergilli tested.

The chemical analysis showed there was an increase in protein and amino acid content of FCSM. This apparently resulted from anabolic processes of fermentation that culminated in massive growth of fungal mycelium. The higher levels of amino acids and proteins in FCSM were most likely responsible for much of the improvements in weight gain and feed efficiency by broiler chicks.

There was a 2.5% decrease in gross energy of CSM as a result of fermentation but this decrease had no detectable adverse effect on weight gain of chicks. The energy loss probably represented the cost of fermentation. Zamora and Veum (1979a) observed a 3% less in dry matter of soybeans fermented with A. oryzae or R. oligosporus. The increase in ADF of 3.5 percentage units observed in this experiment was related inversely to the decrease in gross energy in FCSM. Murata et al. (1967), Quinn et al. (1975) and van Veen et al. (1968) also observed an increase in crude fiber of fermented substrates, and they explained that the crude fiber level increased because the more readily digestible carbohydrates in the substrates were utilized by the fungi. Consequently, crude fiber constituted a higher portion of the remaining dry matter.

Although an increase in α-amino acid content of FCSM may have been largely responsible for weight gain of broilers fed FCSM diets, the evidence is not conclusive. Fungal fermentation also may contribute other beneficial nutritional factors to the final product. Several researchers have indicated that predigestion of fermented product with various fungal enzymes enhanced digestibility and increased the availability of nutrients to the chicks. The beneficial hydrolytic contributions of enzymes such as protease (Plating and Cherry, 1979), amylase (Hesseltine, 1965), cellulase (Herr et al., 1978), and phytase (Rojas and Scott, 1969) have been reported to improve the nutritive value of fungal fermented meals.

Experiment 2. Effect of Unsterilized Fermentation with *A. oryzae* and *A. janus* on the Nutritive Value of Cottonseed Meal

Results

The fermented cottonseed meals (FCSM) tested in experiment 1 were produced by sterilizing CSM prior to fermentation. The FCSM evaluated as protein sources in experiment 2 were produced by fermenting unsterilized CSM (nonasceptic fermentation) with either *A. oryzae* or *A. janus*.

Chemical evaluation Chemical examination of the FCSM (Table 8) revealed that fermentation increased crude protein by 5 percentage units and acid detergent fiber (ADF) by 3.3 percentage units. There was no significant change in crude fiber but the gross energy of FCSM decreased by 1.97

Table 8. Essential amino acids, crude fat, ADF, crude protein and GE[a] content of CSM and unsterilized FCSM (percent of dry matter), experiment 2

Item	Nonfermented CSM	*A. oryzae* FCSM	% Δ[b]	*A. janus* FCSM	% Δ[b]
Crude fat	5.47	5.4	-.07	5.3	-.17
ADF	15.37	18.7	+3.33	18.65	+3.28
GE	4647	4555	-1.97	4524	-2.6
Crude protein	41	46.12	+5.12	46.25	+5.25
Arginine	3.60	3.95	+9.0	4.06	12.7
Histidine	1.02	1.71	+67	1.30	+27
Isoleucine	1.20	1.40	+16	1.59	+32
Leucine	2.02	2.44	+20	2.66	+31
Lysine	1.46	1.78	+22	2.02	+38
Methionine	.58	.77	+32	.78	+36
Phenylalanine	1.92	2.48	+29	2.51	+30.7
Threonine	1.12	1.45	+29	1.51	+34
Tryptophane	-	-		-	
Valine	1.52	1.99	+31	2.16	+42
% α-AA[c] of meal	31.64	34.24	+2.6	37.5	+5.86

[a]GE = gross energy in calories per gram.

[b]Percentage change as compared to nonfermented CSM.

[c]AA = amino acids.

to 2.6% as compared with CSM.

Fermentation significantly ($P \leq .05$) increased the concentration of all the essential amino acids listed in Table 8. The magnitude of increase of each amino acid as a result of fermentation also is shown in Table 8. Total α-amino acids were increased by 2.6 percentage units in FCSM produced with A. oryzae and 5.8 percentage units in FCSM produced with A. janus as compared with α-amino acids in unfermented CSM.

The inclusion of FCSM produced with A. janus in the diet increased dietary lysine concentration by 5.7% and methionine by 20% in comparison with the levels of these amino acids in the CSM diet (Table 9). Also the inclusion of FCSM produced by A. oryzae in the diet increased lysine by 3.5% and methionine by 13% in comparison with the CSM diet.

Biological evaluation The mean weight gain and feed conversion data are shown in Table 10. When unsterilized FCSM was substituted for unfermented CSM in diets fed to broiler chicks, weight gain was improved significantly ($P \leq .05$) (9 to 14%). However, chicks fed diets containing CSM, which was adequately supplemented with lysine and methionine, gained 26% more weight than chicks fed diets containing unsterilized FCSM. Also, chicks fed supplemented CSM diet gained 41% more weight than those fed unsupplemented CSM diet.

There was no significant difference in weight gain between chicks fed diets containing SBM and chicks fed diets containing CSM supplemented with lysine and methionine.

Table 9. Essential amino acid composition of diets (percent of diet), experiment 2

Amino acids	SBM	CSM+L+M[a]	CSM	A. janus (unsterilized)	A. oryzae (unsterilized)
Arginine	1.19	1.80	1.80	1.52	1.49
Histidine	.51	.55	.55	.58	.54
Isoleucine	.92	.65	.65	.69	.64
Leucine	1.89	1.67	1.67	1.70	1.62
Lysine	1.26	1.28	.82	.92	.85
Methionine	.53	.54	.29	.35	.33
Meth + cystine	.82	.83	.58	.55	.67
Phenylanine	1.22	1.12	1.12	1.07	1.13
Threonine	.78	.73	.73	.78	.73
Tryptophane	.24	.23	.23	-[b]	-[b]
Valine	1.07	1.09	1.09	1.12	1.08

[a]M = D·L-methionine (0.25%), L = L-lysine (0.41%).

[b]Analytical method employed oxidized tryptophane.

Table 10. Effect of feeding unsterilized, fermented CSM on weight gain and feed/gain of broiler chicks (7-21 days of age), experiment 2[a]

Diet	Weight gain[b] (g)	% change above CSM[c]	Feed/ gain (g)	Feed consumed/ chick
SBM	328.6 a	+47.6	1.69 d	556 ab
CSM+L+M	313.9 a	+41	1.90 c	595 a
CSM	222.6 c	-	2.46 a	547 ab
FCSM, A. oryzae	253.6 b	+14	2.11 b	534 b
FCSM, A. janus	243.5 b	+9.4	2.21 b	538 b

[a]Means in the same column and with different letters are significantly different ($P \leq .05$).

[b]Mean weight per chick.

[c]Percentage increase in weight gain as compared to CSM diet.

Weight gain, feed consumption and feed conversion were not significantly different ($P \geq .05$) between chicks fed FCSM produced with A. oryzae and chicks fed FCSM produced with A. janus.

Chicks fed diets containing FCSM consumed significantly ($P \leq .05$) less feed than chicks fed diets containing unfermented CSM, supplemented CSM or soybean meal.

Feed efficiency was improved significantly by feeding diets containing FCSM. Chicks fed a diet containing SBM or the supplemented CSM diet, however, had a significantly better

feed efficiency than chicks fed diets containing FCSM. Also, the SBM diet was utilized more efficiently than was the supplemented CSM diet.

Fermented CSM from both species of aspergilli caused no mortality to broiler chicks during the 14-day experiment.

Discussion

In principle, unsterilized fermentation, or nonaseptic fermentation, is carried out by lowering the pH of the substrate. This was followed by massive inoculation of the substrate with the desirable spores that would outgrow any possible contaminants (Riviere, 1977). Since no mortality was observed by feeding diets containing unsterilized FCSM, the CSM used for fermentation was either relatively free of spores of toxic organisms, such as Aspergillus flavus, or(and) the massive inoculation of the CSM with spores of either A. oryzae or A. janus suppressed possible contaminants. This finding is in agreement with that of Reade et al. (1972). They successfully fermented barley with A. oryzae under nonasceptic condition at pH 3.5. The fermentation products were fed to rats and pigs with no ill effects.

Diener et al. (1963) found that A. flavus in fermented peanut produced toxins which killed ducklings. Diener and Davis (1970) observed that when A. flavus was present in a culture at 30°C, it would take 21 days to produce large quantities of aflatoxin. This suggests that if spores of a toxic

fungus were present in CSM, the 48 hours of fermentation in this experiment was not long enough for substantial amounts of toxins to develop.

The improved weight gain and feed efficiency observed in this experiment when FCSM was fed was probably the result of an increase in the α-amino acid content of FCSM. The 9 to 14% improvement in weight gain observed by feeding diets containing FCSM was in agreement with the finding of Chah et al. (1976a). They observed 9 to 12% improvement in weight gain when diets containing fermented soybeans were fed to broiler chicks.

The improved feed efficiency observed when diets containing FCSM were fed indicated that FCSM was more efficiently utilized than diets containing unfermented CSM. The improved feed efficiency was partly due to higher amino acid content of FCSM. Additionally, the catabolic processes of fermentation may have been beneficial too. Fungi digest the CSM extracellularly to be able to absorb the nutrients. This process of predigestion might make the nutrients in FCSM more available than they were originally in CSM, and of course, fungal protein is of high quality and readily digested (Imrie, 1973).

The results obtained from experiments 1 and 2 suggest that sterilization of CSM prior to fermentation was not necessary. Nonsterilized FCSM was higher in essential amino acids

and crude fat than sterilized FCSM. Chicks seemed to utilize diets containing unsterilized FCSM more efficiently (experiment 2) than they did diets containing sterilized FCSM (experiment 1). Unsterilized CSM, acidified to a pH of 3.5, was just as successfully fermented as sterilized FCSM. Diets containing unsterilized FCSM or sterilized FCSM did not cause mortality to the chicks. There was only one advantage in feeding sterilized FCSM. The relative magnitude of response in weight gain with chicks fed diets containing sterilized FCSM was higher than that observed with chicks fed diets containing unsterilized FCSM.

It seems only necessary to sterilize the substrate used in the multiplication of spores (source of inoculum). This is necessary for the production of pure and virile spores for the mass inoculation of an acidified, production batch of CSM. A similar approach is employed in the cheese industry (Kavaler, 1972). The substrate for cheese production is pasteurized prior to fermentation but the product of fermentation is not sterilized. Sterilization is an expensive process requiring much energy and time. It may not be economical to sterilize CSM prior to solid state fermentation with A. oryzae or A. janus.

Experiment 3. Lysine and Methionine as the Critical Amino Acids in Fermented Cottonseed Meal and Cottonseed Meal Diets

Results

In experiment 1, the inclusion of fermented cottonseed meal (FCSM) in the diet improved weight gain per chick by 32 to 35 g as compared to gains of chicks fed negative cottonseed meal (CSM) diet. Because the magnitude of improvement was about 50% of that observed when CSM was fully supplemented with lysine and methionine to meet NRC (1977) requirements (supplemented CSM diet), a third experiment was conducted to test the hypothesis that fermentation improved chick weight gain primarily by increasing lysine and methionine content of CSM. The amino acid analysis of FCSM (Table 8) in experiment 2 and the resulting dietary amino acid levels of the FCSM diet (Table 11) support this hypothesis.

The unsupplemented CSM diet contained 0.82% lysine and 0.29% methionine (Table 11). This was the negative control diet. The positive control CSM diet was supplemented with 0.41% L-lysine and 0.25 D·L-methionine and contained 1.28% lysine and 0.54% methionine. In experiment 3, the diet containing FCSM was partially supplemented with 0.205% L-lysine and 0.125% D·L-methionine and contained 1.00% lysine and 0.46 methionine. This supplementation was one-half of lysine and methionine needed to meet NRC (1977) requirements of CSM diet.

Table 11. Amino acid composition[a] of diets supplemented with lysine and methionine, experiment 3

Amino acid	CSM	CSM +L+M	A. oryzae +½L+½M[b]	A. oryzae +½L+½M[c]	A. oryzae +M[c]
Arginine	1.80	1.80	1.53	1.49	1.49
Histidine	.55	.55	-[d]	.54	.54
Isoleucine	.65	.65	.63	.64	.64
Leucine	1.67	1.67	1.55	1.62	1.62
Lysine	.82	1.28	1.00	1.05	.85
Methionine	.29	.54	.54	.46	.58
Meth + cystine	.58	.83	1.05	.80	.92
Phenylalanine	1.12	1.12	1.09	1.13	1.13
Threonine	.73	.73	.73	.73	.73
Tryptophan	.23	.23	-[d]	-	-
Valine	1.09	1.09	.90	1.08	1.08

[a]Amino acid as percent of diet.

[b]The diet was analyzed for the amino acids.

[c]Amino acid values of the diets were calculated from amino acid analysis data of FCSM and other ingredients used in the diet. The levels of lysine and methionine supplementation were: M = D·L-methionine (0.25%), ½M = D·L-methionine (0.125%), L = L-lysine (0.41%), ½L + L-lysine (0.205%).

[d]Analytical procedures oxidized histidine and tryptophane.

The mean weight gain, feed conversion and feed consumption data are shown in Table 12. Weight gains were not significantly different ($P \geq .05$) between chicks fed the fully supplemented CSM diet and those fed the partially supplemented FCSM diet. The partial supplementation was with one-half of the lysine and methionine needed to meet NRC (1977) requirements of CSM diet. However, feed efficiency was significantly better ($P \leq .05$) for chicks fed partially supplemented FCSM diets than for chicks fed the fully supplemented CSM diet. Chicks fed the fully supplemented CSM or partially supplemented FCSM diets, however, gained significantly less weight and utilized feed less efficiently than did chicks fed the soybean meal diet.

There were no significant differences ($P \geq .05$) in amounts of feed consumed among chicks fed the partially supplemented FCSM diet and those fed the fully supplemented CSM or soybean meal diets.

An attempt was made to determine whether methionine was the first limiting amino acid in FCSM diet. This is based on the principle that the nutritive value of an imbalanced diet can be improved by supplementing the most limiting amino acid. Fermented CSM, produced with A. oryzae, was used in a diet which was fully supplemented with D·L-methionine (.25%) alone. This supplementation provided 0.58% methionine or 0.92% total sulfur amino acids (TSAA) (Table 11) in the diet. Chicks fed the diet containing FCSM, supplemented with

Table 12. Effect of amino acid supplementation of fermented CSM on weight gain and feed efficiency of broiler chicks (7-28 days), experiment 3[a]

Diet	Weight gain[b] (g)	% change above CSM	Feed/ gain (g)	Feed consumed/ chick (g)
SBM	592.7 a	+25.5	1.76 d	1044 a
CSM+L+M[c]	534.3 b	+13.2	2.02 b	1074 a
CSM	471.9 c	-	2.17 a	1023 a
FCSM, A. oryzae +½L+½M[d]	540.3 b	+14.5	1.90 c	1029 a
FCSM, A. janus +½L+½M	528.0 b	+12	1.92 c	1015 a
FCSM, A. oryzae+M	421.2 d	-10.7	2.21 a	931 b

[a]Means in the same column and with different letters are significantly different ($P \leq .05$).

[b]Mean weight gain per chick.

[c]M = D·L-methionine (.25%), L = L-lysine (.41%).

[d]½M = D·L-methionine (.125%), ½L = L-lysine (.205%).

methionine only, gained significantly ($P \leq .05$) less weight than those fed the unfermented, unsupplemented, CSM negative control diet (Table 12). The magnitude of the reduced weight gain was -10.7%. Also, feed consumption was reduced by 9% as compared with the unsupplemented CSM diet. Feed conversion, however, did not differ significantly ($P \geq .05$) between chicks fed unsupplemented CSM diet and those fed methionine supplemented FCSM diet.

Discussion

The levels of lysine and methionine supplementation in the CSM diet were those needed to meet the NRC (1977) requirements. But the FCSM diets were partially supplemented with one-half of lysine and methionine needed to meet the NRC (1977) requirements of CSM diet. There were no significant differences in weight gain between the chicks fed partially supplemented FCSM diet and those fed fully supplemented CSM diet. Because the level of FCSM used in the diet was lower than the level of unfermented CSM used in supplemented CSM diet, the results show that FCSM contained higher levels of lysine and methionine than unfermented CSM. Also, the data presented in Table 11 showed that lysine and methionine levels in FCSM diets and in the supplemented CSM diet were higher than those in the CSM diet. But the levels of other essential amino acids in FCSM diets were not higher than those in CSM or supplemented CSM diets. This suggests that lysine and methionine were mainly responsible for the improved weight gain and feed efficiency observed when FCSM diets were fed to broiler chicks.

Quantitatively, fermentation seemed to result in the synthesis and/or improved availability of lysine and methionine equivalent to about 50% of the amounts of these two amino acids needed as supplements in an unfermented CSM diet. The amino acid analysis data support the former but increased amino acid availability should not be overlooked because Imrie (1973) observed a high digestibility of fungal

protein and a high availability of lysine from fungal protein.

Feed conversion was significantly better on the unsupplemented FCSM diet than on the supplemented CSM diet. This suggests that FCSM diet was utilized more efficiently than was the supplemented CSM diet, probably because the lysine and methionine (and certain other nutrients) in FCSM were more available to the chicks.

Chah et al. (1976a) reported a similar observation when they supplemented a soybean control diet with several amino acids to simulate the amino acids in fermented soybean diet. They found that, although the soybean control diet contained the same level of amino acids as the fermented soybean diet, the fermented soybean diet was significantly better in supporting weight gain and feed efficiency.

The response to methionine supplementation in FCSM diet was negative. This finding is in agreement with the work of Smith et al. (1975) who found that, when a rat diet containing the mycelium of A. oryzae was supplemented with L-methionine, net protein utilization did not increase. The data in table 12 indicated that methionine was not the first limiting amino acid in a diet containing FCSM produced with A. oryzae. This is in agreement with the finding of Harper et al. (1970). They reported that the addition of a second most limiting amino acid to a diet would cause amino acid imbalance in the absence of the first limiting amino acid. The data reported

herein show that methionine is probably a second limiting amino acid in FCSM diet. The reduced weight gain and lowered feed consumption indicated an amino acid imbalance and/or methionine toxicity as a result of excess methionine in the FCSM diet supplemented with this amino acid.

Experiment 4. Effect of Ammonium Sulfate Enriched, Fermented Cottonseed Meal on Growth of Broilers

Introduction

The hypothesis tested in this experiment was that the fermenting organism, A. oryzae, would utilize the nitrogen and sulfur of ammonium sulfate (AS) and the carbohydrate of cottonseed meal (CSM) to synthesize amino acids and proteins, thereby enhancing the nutritional value of CSM.

Ammonium sulfate was included in CSM prior to fermentation with A. oryzae. The levels of AS used were 1, 2, 3, 4 and 5%, by weight, in CSM. The CSM was not sterilized (non-aseptic) prior to fermentation and contained 33% added water. The CSM, enriched with AS, was fermented at 30°C for 48 hours and was processed for feeding as described previously. There were two positive control diets. One was a CSM diet that was partially supplemented with one-half of lysine and methionine needed to meet the NRC (1977) requirements. A second control diet contained soybean meal as the major protein source. A negative control diet contained CSM

as the major protein source.

Results

With the exception of arginine, all the products of fermentation contained higher levels of essential amino acids than nonfermented CSM (Table 13). There were no significant changes in essential amino acid content of FCSM enriched with 1, 2, or 3% AS as compared with FCSM containing no AS. Fermented FCSM, enriched with 4 to 5% AS, contained significantly lower levels of essential amino acids than FCSM enriched with 0, 1, 2 or 3% AS.

The FCSM enriched with 1, 2, or 3% AS contributed more methionine and threonine to the diets (Table 14) than FCSM containing 0% AS. All the FCSM, irrespective of the level of AS, contributed significantly ($P \leq .05$) more methionine to the diets than unfermented CSM. But the amount of FCSM included in the diet was lower than the amount of CSM used in the negative CSM control diet. Enriched FCSM containing 1, 2 or 3% AS contributed lysine levels which were not significantly different from the amount of lysine contributed by FCSM with 0% AS. Fermented cottonseed meal, enriched with 4 or 5% AS, contributed less lysine and threonine to the diets than FCSM enriched with 1, 2 or 3% AS.

Crude protein Crude protein level of enriched FCSM increased with increasing level of AS of the meal. This linear increase in crude protein probably reflected an

Table 13. Essential amino acids, crude fat and crude protein content of ammonium sulfate (AS) enriched fermented CSM (percent of dry matter), experiment 4

Item	Non-fermented CSM	*A. oryzae* fermented CSM, AS levels					
		0	1	2	3	4	5
Crude fat	5.47	5.40	4.80	4.25	4.50	4.00	4.40
Crude protein	41	46.12	47.50	48.90	49.80	52.25	53.25
Arginine	3.76	3.95	3.70	4.04	4.15	3.60	3.40
Histidine	1.02	1.70	-[a]	-	-	-	-
Isoleucine	1.20	1.40	1.36	1.35	1.40	1.28	1.25
Leucine	2.02	2.44	2.46	2.38	2.48	2.28	2.22
Lysine	1.46	1.78	1.81	1.74	1.75	1.64	1.55
Methionine	.58	.77	.74	.74	.75	.68	.66
Phenylalanine	1.92	2.48	2.54	2.30	2.51	2.21	2.00
Threonine	1.12	1.45	1.45	1.38	1.43	1.24	1.28
Tryptophane	.23	-[a]	-	-	-	-	-
Valine	1.52	1.99	1.80	1.78	1.80	1.64	1.60
% α-AA of meal	31.60	34.20	33.80	32.30	34.20	30.80	30.70

[a]Histidine and tryptophane oxidized by analytical method.

Table 14. Essential amino acid composition of diets (percent of dry matter), experiment 4

Amino acids	SBM	CSM +½L+½M[b]	% AS[a] levels of FCSM diets					
			0	1	2	3	4	5
Arginine	1.19	1.80	1.49	1.42	1.52	1.55	1.40	1.34
Histidine	.51	.55	.54	-[c]	-	-	-	-
Isoleucine	.92	.65	.64	.63	.63	.64	.61	.60
Leucine	1.89	1.67	1.62	1.65	1.62	1.65	1.60	1.58
Lysine	1.26	1.03	.85	.86	.84	.84	.81	.79
Methionine	.53	.41	.33	.34	.34	.34	.32	.31
Phenylalanine	1.22	1.12	1.13	1.15	1.08	1.14	1.06	1.00
Threonine	.78	.73	.73	.76	.70	.75	.70	.71
Tryptophane	.24	.23	-[c]	-	-	-	-	-
Valine	1.07	1.09	1.08	1.03	1.02	1.03	.98	.97

[a]AS = ammonium sulfate.

[b]½L = L-lysine (.205%), ½M = D·L-methionine (.125%).

[c]Histidine and tryptophane oxidized by analytical method.

accumulation of inorganic nitrogen (AS).

Crude fat There was no significant difference in the level of crude fat in FCSM enriched with 0 and 1% AS as compared with unfermented CSM. But, FCSM enriched with 2, 3, 4, or 5% AS contained significantly less crude fat than unfermented CSM.

Biological evaluation Broiler chicks fed diets containing FCSM enriched with 0, 1 or 2% AS, gained significantly ($P \leq .05$) more weight than chicks fed diets containing FCSM enriched with 3, 4 and 5% AS (Table 15).

At 4 weeks of age, chicks fed diets containing FCSM, irrespective of the level of AS, gained significantly more weight than chicks fed diets containing unfermented CSM.

There was no significant difference in weight gain of chicks fed diets containing FCSM enriched with 0% AS and those fed diets containing FCSM enriched with 1 or 2% AS. At 2, 3, and 4 weeks of age, however, the mean weight gain of chicks fed diets containing FCSM, enriched with 2% AS, was consistently higher than the mean weight gain of chicks fed diets containing FCSM enriched with 0 or 1% AS.

Diets containing FCSM, enriched with 3, 4 or 5% AS, depressed weight gain of chicks as compared with the diet containing FCSM with 0% AS.

There was no significant difference in weight gain among chicks fed diets containing FCSM with 0, 1 or 2% AS and those fed diets containing partially supplemented CSM diet. The

Table 15. Effect of ammonium sulfate (AS) enriched, fermented CSM on growth and feed/gain of broilers at 4 weeks of age, experiment 4[a]

Diets	Weight gain[b] (g)	Feed/ gain (g)	Feed consumed/ chick
SBM	543.4 a	1.80 d	988 ab
CSM+½L+½M[c]	461.4 b	2.15 bc	992 ab
CSM	367.8 f	2.41 b	885 b
FCSM+0% AS[d]	473.8 b	2.10 c	999 a
FCSM+1% AS	474.2 b	2.15 bc	1019 a
FCSM=2% AS	483.8 b	2.09 c	1014 a
FCSM+3% AS	443.8 c	2.22 bc	986 ab
FCSM+4% AS	407.0 d	2.35 bc	960 ab
FCSM+5% AS	382.2 e	2.67 a	1024 a

[a]Means in the same column and with different letters are significantly different ($P \leq .05$).

[b]Mean weight gain per chick.

[c]½L = L-lysine (.205%), ½M = D·L-methionine (.125%).

[d]FCSM = fermented cottonseed meal.

partially supplemented CSM diet contained one-half of the supplemented lysine and methionine needed to meet NRC (1977) requirements.

Regression analysis of weight gain versus level of AS (Figure 2) showed that the linear and quadratic trends were significant ($P \leq .05$). The prediction equations for 7 to 14,

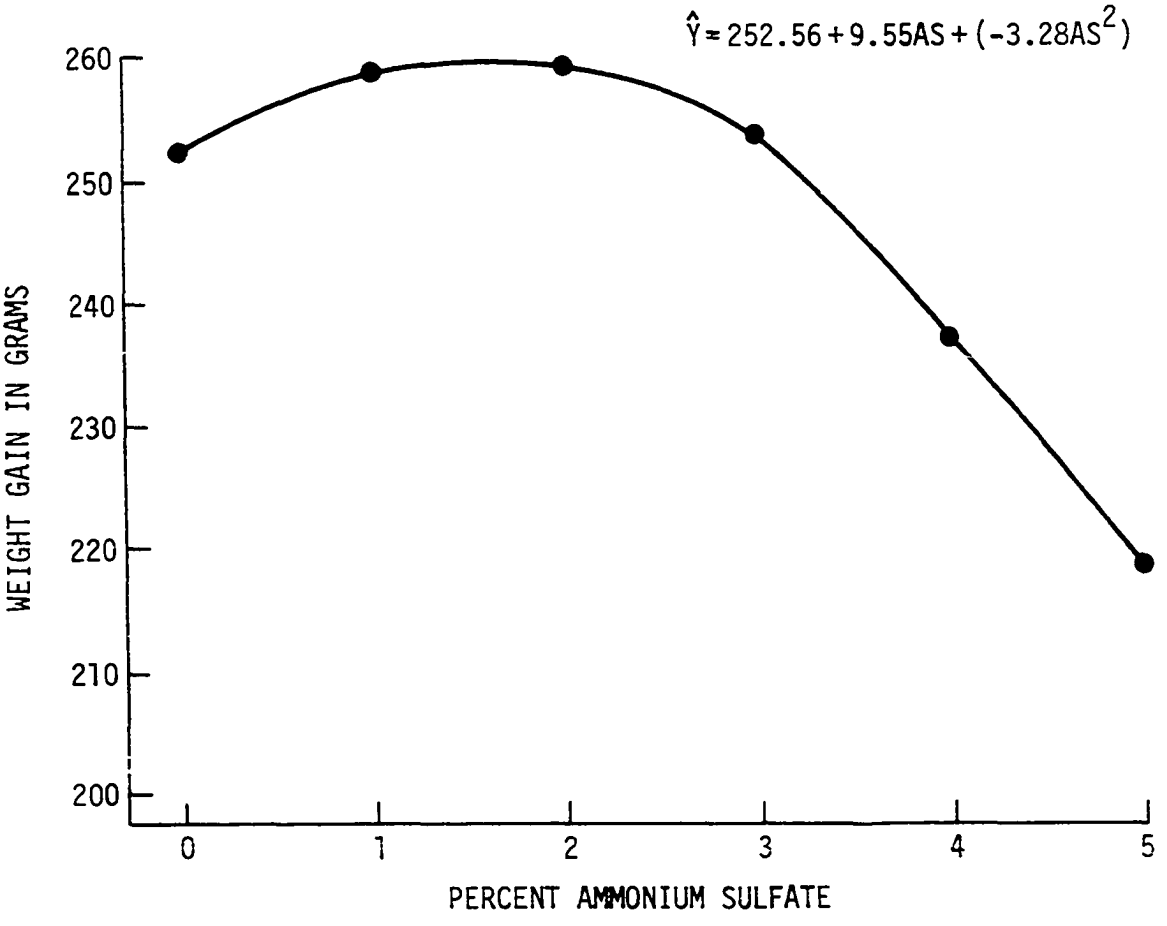

Figure 2. Regression equation relating body weight gain of chicks (7-21 days of age) to percent AS used in preparation of FCSM, experiment 4

7 to 21 and 7 to 28 days of age cumulative weight gain per chick were as follows:

Model: $\hat{Y} = b_0 + b_1X + b_2X^2$, where $\hat{Y}$ predicted weight gain, b_0 intercept, b_1X = linear component, b_2X^2 = quadratic component, and X = dependent variable or AS.

7 to 14 days: Weight gain = 108 + (-0.494AS) + $(-0.758AS^2)$.

7 to 21 days: Weight gain = 252.56 + 9.56AS + $(-3.288AS^2)$.

7 to 28 days: Weight gain = 475.676 + 7.6AS + $(-5.49AS^2)$.

According to the prediction equation, FCSM enriched with 1.5% AS was optimum for weight gain at 3 weeks of age. Also, FCSM with 0.7% AS was optimum for weight gain at 4 weeks of age. The data in Table 15, however, indicated that chicks fed diets containing FCSM, enriched with 2% AS, attained the heaviest weight as compared with chicks fed diets containing FCSM enriched with 1% AS, but the difference was not statistically significant.

Feed consumption Chicks fed diets containing FCSM enriched with 3 or 4% AS consumed nonsignificantly less feed than those fed diets containing FCSM enriched with 1 or 2% AS. Chicks fed diets containing FCSM, enriched with 0, 1, 2 or 5% AS, consumed significantly more feed than the chicks fed diets containing unfermented CSM.

Feed efficiency Feed efficiency was not significantly different for chicks fed diets containing FCSM with 0% and 2% AS. These diets were not more efficiently utilized than the diet containing partially supplemented CSM.

Chicks fed diets containing FCSM enriched with 0 and 2% AS had significantly better feed efficiency than chicks fed diets containing unfermented CSM. Feed efficiency was poorer for chicks fed diets containing FCSM enriched with 5% AS than for those fed diets containing unfermented CSM.

Discussion

It was shown in experiment 3 that additional lysine and methionine formed during fermentation were mainly responsible for growth improvement when diets containing FCSM were fed to broiler chicks. In this experiment, lysine and methionine contents of FCSM, enriched with 1, 2 and 3% AS, were not changed significantly as compared with their concentration in FCSM not enriched with AS. Chicks attained a maximum weight on diets containing FCSM enriched with 2% AS. The improved weight gain with increasing level of 1 to 2% AS in FCSM suggests that the fungi may have changed slightly the quality and profile of essential amino acids rather than increasing their levels per se. The major pathway for the formation of α-amino acid groups directly from ammonia (NH_3) of AS is through the formation of glutamate from α-ketoglutarate:

NH_3 + α-ketoglutarate + NAD(P)H + H → L-glutamate + NAD(P) + H_2O (Lehninger, 1977). Subsequently, the α-amino group of the glutamate is transferred to various α-keto acids, including those of essential amino acids, to yield the corresponding essential amino acid.

The mean weight gain of chicks increased with increasing level of AS in FCSM up to 2% AS. This trend also indicated that some AS was converted to high quality protein. Similar observations were made by Reade et al. (1972) when starch of barley was hydrolyzed to glucose by enzymes of A. oryzae. The glucose was assimilated together with ammonium sulfate nitrogen for synthesis of fungal protein. Reade et al. (1972) used 0.42% AS with 3% barley in submerged non-aseptic fermentation.

Weight gained by chicks fed diets containing FCSM, enriched with 0, 1 or 2% AS, was not significantly different from weight of chicks fed partially supplemented CSM diet. This finding confirms the earlier observation (experiment 3) that fungal fermentation resulted in higher levels and/or improved availability of lysine and methionine. Quantitatively, the change in lysine and methionine was equivalent to about 50% of the amounts of these amino acids needed as supplements in the CSM diet to meet NRC (1977) requirements.

A significant decline in total α-amino acids in FCSM enriched with 4 and 5% AS indicates that these higher levels

of AS impaired fungal growth. The impairment may have resulted from the acidity caused by the sulfate moiety of AS. Semeniuk (1938) explained that NH_4^+ was more available to the fungus and AS made the culture acidic due to the resulting accumulation of the $SO_4^=$ radical. Consequently, the culture was rendered less favorable for growth of the fungus.

The reduction in weight of chicks when FCSM enriched with more than 2% AS was included in the diets suggests that higher levels of AS also may be toxic to chicks. The decline in amino acid levels of FCSM enriched with 4 and 5% AS could explain part of the reduction in weight gain of chicks. But there may have been sufficient residual AS in the FCSM enriched with 3 to 5% AS to impair chick growth. Bell and Freeman (1971) indicated that excess NH_4^+ might be toxic to chicks because they lack the enzyme carbamyl phosphatase which could convert $NH_3 + CO_2 \xrightarrow[\text{Biotin}]{2ATP \quad 2AAP}$ carbamyl phosphate for urea cycle. Excretion of ammonia nitrogen in chicks accounts for only 10 to 15% of total nitrogen excreted (Sturkie, 1976). This could explain part of the reduction in weight gain observed when FCSM enriched with 3, 4 and 5% AS was included in the diets.

Chicks fed diets containing FCSM enriched with 1 to 5% AS consumed more feed than chicks fed diets containing unfermented CSM. This finding is contrary to that of Sibbald

and Cave (1976). Their diet contained no fermentation products. They found that diets containing 1 to 5% AS caused a decline in feed consumption and suggested that AS could be used to reduce voluntary feed intake of chicks. The lack of similar effect in this experiment indicates that during fermentation some of the AS may have been converted to fungal protein and/or other substances that were less detrimental to feed consumption.

GENERAL DISCUSSION

Fungal proteins belong to a group of proteins called single cell proteins. Single cell protein, and its acronym SCP, is a generic term for crude or refined sources of protein whose origin is unicellular or simple multicellular organisms such as bacteria, yeast, fungi, algae, and protozoa (Tannenbaum, 1976). Fungal proteins are produced by fermentation, which is a process of decomposing or rearranging organic substances with a self-multiplying microbial catalyst or enzyme (ferment); the microbial cell is itself one of the products of the process. Any fungal fermented food will contain significant quantities of fungal cells (Pederson, 1971). Hence, the fermented CSM (FCSM) produced during the research reported here was a conglomeration of modified or predigested CSM, fungal protein and fungal metabolites. The FCSM was evaluated as a new protein source for broiler chicks. The parameters evaluated were nutrient change (protein, amino acid, crude fat, crude fiber, and gross energy) in CSM as a result of fermentation and the effects of dietary FCSM on weight gain, feed efficiency and mortality of chicks. This general discussion includes the method of solid state fermentation and the evaluation of the nutritive potential of FCSM.

Solid State Fermentation

The regulation of water, temperature and ventilation was crucial for adequate fermentation of CSM. The term, solid state, implies that the fermentable substrate does not contain excess water to merit the term semi-solid (slurry) or liquid state as in submerged fermentation with bacteria or yeast. In this experiment, 33% added water was adequate for CSM fermentation, but Trevelyan et al. (1974) found 47% added water to be adequate for the fermentation of cassava meal. The mixture of cassava and water was made into a dough and extruded to provide for adequate aeration and increased surface area. In the current research, the CSM wetted with 33% added water was thinly spread (1 to 2 cm deep) to provide for adequate aeration. The vents in plastic bags and aluminum foil provided for dissipation of excess heat to control temperature.

Unlike cracked grains, CSM has no granular structure when it is wet and it is easily compacted to occlude air. The fine meals, dust, and excess water occluded air spaces. According to Semeniuk et al. (1970), cracked grain was screened free of fine dust particles before it was soaked in water. The cracked, soaked grains or soybeans were cooked and the water was drained prior to fermentation (Hesseltine et al., 1967; Chah et al., 1975). According to Chah et al. (1975), cracked soybeans were conditioned to

about 31% moisture prior to inoculation. Optimum level of added water is crucial to adequate aeration in solid state fermentation, especially meals containing very fine dust.

There was no agreement among several authors on the duration of fermentation. The duration of fermentation varied from 18 hours (Zamora and Veum, 1979a) to 6 days (Semeniuk et al., 1970) for soybeans. A period of 48 hours was adequate in this experiment. This seemed reasonable because Kihlberg (1972) reported that filmentous fungi can double their masses in 4 to 12 hours during fermentation.

Most workers sterilized their cracked grains or soybeans prior to fermentation. Probably cooking was a necessary step to gelatinize the starch and soften the grain for fungal penetration. In the research reported here, unsterilized, acidified (pH 3.5) CSM supported copious fungal growth comparable to the growth observed on sterilized CSM. This finding is in agreement with the observation of Van Veen et al. (1968) that omitting sterilization of peanut cake before inoculation did not affect mold growth appreciably.

Nutrient Change

In experiments 1 and 2, it was found that fermentation of sterilized or unsterilized CSM resulted in increased crude protein, essential amino acids, acid detergent fiber (ADF) concentrations as compared with unfermented CSM. This finding is in agreement with the observation of Quinn et al.

(1975) with fermented peanut flour. The increase in protein and amino acids was a result of synthesis of fungal proteins. Also, Quinn et al. (1975) and van Veen et al. (1968) observed an increase in crude fiber of fermented peanut cake and peanut flour. They explained that the crude fiber level increased because the more readily digestible carbohydrates in the substrates were utilized by the fungi. Consequently, crude fiber constituted a higher portion of the remaining dry matter.

There was no change in crude fat concentration of unsterilized FCSM as compared with crude fat content of unfermented CSM. van Veen et al. (1968) observed a similar trend with fermented peanut cake. But, in the current research, there was a decrease in crude fat content of sterilized FCSM as compared with unfermented CSM. Perhaps, the process of autoclaving CSM volatilized some lipid component of CSM. This could have resulted in a lower crude fat content of sterilized FCSM.

The gross energy of sterilized FCSM and unsterilized FCSM decreased significantly ($P \leq .05$) as a result of fermentation. The gross energy probably decreased because the fungus utilized some energy for its metabolism and mycelial development. There usually is a 3 to 10% loss in dry matter as a consequence of energy utilization by the fermenting agent (Whitaker, 1978; Zamora and Veum, 1979a). The loss in gross energy of 1.7 to 3% in this experiment did not affect weight gain or feed efficiency of broilers adversely as

compared with the performance of chicks fed unfermented CSM.

Experiment 4 was conducted to determine the effect of enriching CSM with ammonium sulfate (AS) before fermentation on the nutritive value of the FCSM. Ammonium sulfate was added to CSM to provide levels of 1, 2, 3, 4, and 5% in the substrate. The enriched CSM was fermented with A. oryzae.

There was no significant difference ($P \geq .05$) between amino acid contents of nonenriched FCSM and FCSM enriched with 1, 2, and 3% AS. But amino acid contents of FCSM enriched with 4 and 5% AS decreased significantly as compared with amino acid contents of nonenriched FCSM. Apparently, excess AS was toxic to A. oryzae and depressed synthesis of essential amino acids. Hence, the amino acid contents of FCSM was not increased by enriching CSM prior to fermentation with A. oryzae. Probably, CSM was high enough in nitrogen for the fungus, and an additional source of nitrogen was not beneficial. van Veen et al. (1968) reported that addition of 1% tapioca accelerated fungus growth very much in fermented peanut cake. This implies that the quality of vegetable proteins might be improved by supplementing with a source of energy rather than a source of nitrogen.

Weight Gain and Feed Efficiency

Chicks fed diets containing sterilized or unsterilized FCSM gained significantly ($P \leq .05$) more weight and utilized feed more efficiently than chicks fed diets containing unfermented CSM. The improved weight gain was the result of increased protein and amino acid concentration and a more balanced amino acid profile of FCSM. Chah et al. (1976a) also found that the growth stimulation in chicks was a function of superior amino acid balance of fermented soybean diet. Chicks fed diets containing sterilized FCSM gained an average of 29% more weight than chicks fed diets containing unfermented CSM. Zamora and Veum (1979a) also observed a 30% increase in weight gain of rats fed diets containing 20 to 26% fermented soybeans. Chicks fed diets containing unsterilized FCSM gained 9 to 14% more weight than chicks fed diets containing unfermented CSM. This is in agreement with the observation of Chah et al. (1976a) who observed a 7 to 12% increase in weight gain of broilers fed diets containing fermented soybeans produced by any of six species of Aspergilli. But their soybeans were sterilized before fermentation.

Although the magnitude of improvement was larger for chicks fed sterilized FCSM, overall weight gain was higher for chicks fed unsterilized FCSM than chicks fed sterilized FCSM.

There was no significant difference in weight gain of chicks fed diets containing nonenriched FCSM and those fed diets containing FCSM enriched with 1 or 2% AS. This implies that AS was not effective in significantly improving the nutritive value of FCSM. Diets containing FCSM enriched with 2% AS caused only a 2% increase in weight gain as compared to diets containing nonenriched FCSM. This slight increase may not be sufficient for the enrichment of CSM with AS before fermentation to be economical.

The feeding value of a diet containing FCSM enriched with 0, 1 or 2% AS, however, was equivalent to the feeding value of a CSM diet supplemented with one-half of the lysine and methionine needed to meet NRC (1977) requirements. But diets containing FCSM enriched with 3, 4 and 5% AS depressed weight gain of chicks as compared with diets containing nonenriched FCSM.

Amino Acid Supplementation

Experiments 3 and 4 indicated that lysine and methionine were mainly responsible for improving growth of broilers. In experiment 3, one-half of supplemental lysine and methionine needed to increase the dietary levels of the amino acids to meet NRC (1977) requirements in the CSM diet were added to diets containing FCSM. The response of chicks on this partially supplemented FCSM diet was compared to the response of chicks fed CSM diets fully supplemented with

enough lysine and methionine to meet NRC (1977) requirements. There was no significant difference ($P \geq .05$) between weight gain of chicks fed the fully supplemented CSM diet and weight gain of chicks fed the partially supplemented FCSM diets. This result was also duplicated in experiment 4, where the control CSM diet was supplemented with one-half of lysine and methionine needed to meet NRC (1977) requirements. It was concluded that the nutritive value of FCSM was equivalent to CSM with one-half of the supplemental lysine and methionine needed to meet NRC (1977) requirements. This saving would be equivalent to 1.86 kg lysine and 1.13 kg methionine per ton in diets containing FCSM as compared to diets containing CSM.

Chah et al. (1976a) employed a similar approach to prove that the increases in several amino acids were responsible for growth improvement when diets containing fermented soybeans were fed to chicks. They supplemented a soybean control diet with several amino acids to simulate the amino acid contents of fermented soybean diet.

The negative response to methionine supplementation observed in experiment 3 showed that lysine was the first limiting amino acid and methionine was the second limiting amino acid in the FCSM diet. This is in agreement with the observation of Harper et al. (1970) who reported that the addition of a second most limiting amino acid to a diet would cause amino acid imbalance in the absence of the first limiting amino acid. Reduced weight gain, lowered feed consumption,

and poor feed efficiency were observed on FCSM diet supplemented with methionine as compared to CSM diet. Hence, lysine and methionine need to be supplemented together in FCSM diets to prevent an amino acid imbalance.

Products of A. oryzae and A. janus Compared

There was no significant difference in the nutritive value of FCSM produced with A. oryzae and A. janus. Weight gain, feed efficiency and feed consumed were not significantly different ($P \geq .05$) between chicks fed diets containing FCSM produced with A. oryzae and chicks fed diets containing FCSM produced with A. janus. Hence, either species of Aspergillus could be used to produce FCSM. Quinn et al. (1975) observed that there was no significant difference in weight gain of rats fed diets containing fermented peanut flour produced with A. oryzae and A. elegans.

Mortality

There was no mortality for the period of the experiments when diets containing sterilized FCSM or nonsterilized FCSM were fed to chicks. It was concluded that the products of A. oryzae and A. janus fermentation were not toxic to chicks whether fermentation was done with sterilized or unsterilized CSM. But Chah et al. (1976a) found that fermented soybeans produced with certain species of spergilli, A. fisher NRRL185, A. tamarii NRRL434, and A. elegans NRRL4850,

depressed weight gain of chicks as compared with unfermented soybeans. The finding in the experiment reported herein is in agreement with those of Reade et al. (1972). They fermented barley with A. oryzae under nonaseptic condition at pH 3.5. The fermentation products were fed to pigs without ill-effects.

SUMMARY AND CONCLUSIONS

A method for solid state fermentation was developed and employed to produce fermented cottonseed meal. Cottonseed meal (CSM) was fermented with Aspergillus oryzae NRRL506 and Aspergillus janus NRRL1935 as a means of improving the nutritional value of CSM. Some CSM was enriched with 1 to 5% ammonium sulfate (AS) prior to fermentation with A. oryzae. The fermented products were evaluated as new protein sources for broiler chicks.

Fungal fermentation significantly increased the essential amino acid, crude protein and acid detergent fiber of CSM as compared with unfermented CSM. Diets containing sterilized FCSM significantly improved weight gain by 28 to 30% while diets containing unsterilized FCSM improved weight gain by 9 to 14% as compared to diets containing unfermented CSM.

Lysine and methionine may have been responsible for the improved weight gain and feed efficiency in diets containing FCSM. The use of FCSM in diets spared about one-half of supplemental lysine and methionine needed to meet NRC (1977) requirements in a CSM diet.

There seemed to be no obvious advantage in enriching CSM with AS before fermentation. Addition of 1, 2 or 3% AS to CSM before fermentation did not significantly change the essential amino acid content of the enriched FSM as compared

with nonenriched FCSM. Essential amino acid contents of FCSM, enriched with 4 and 5% AS, were significantly lower than in nonenriched FCSM.

Diets containing FCSM, enriched with 1 or 2% AS improved weight gain and feed efficiency, but not significantly, as compared with diets containing FCSM with no AS. The data suggest that CSM (41% protein) contained enough nitrogen for the fungus to use in synthesizing amino acids and proteins. Ammonium sulfate may be more beneficial if it is used to enrich a low protein ingredient prior to fermentation. It is not beneficial to enrich CSM with more than 2% AS prior to fermentation.

Conclusions

1. Cottonseed meal requires 33% added water before solid state fermentation with Aspergillus oryzae or Aspergillus janus.

2. Perforated plastic bags and shallow, perforated trays covered with perforated aluminum foil are suitable for adequate fermentation of cottonseed meal. These modified containers ensure adequate ventilation and conserve enough moisture for optimum fermentation. Trays should not be covered in fermentation chambers with humidity of over 90%.

3. It is not necessary to sterilize cottonseed meal (CSM) prior to fermentation provided the CSM is acidified

(pH 3.5) and inoculated with a mass of fungal spores.

4. Diets containing fermented CSM (FCSM) required only one-half of lysine and methionine supplementation needed otherwise to meet NRC (1977) requirements of chicks fed CSM diet. This saving is equivalent to 1.83 kg lysine and 1.13 kg methionine per ton in diets containing FCSM as compared to diets containing CSM.

5. Diets containing FCSM should not be supplemented with methionine alone because this results in an amino acid imbalance. Both lysine and methionine should be supplemented in FCSM diet to obtain an adequate amino acid balance.

6. It may not be economical to enrich CSM with ammonium sulfate (AS) before fermentation. Weight gain improved by only 2% when diets containing FCSM enriched with 2% AS were fed as compared with feeding diets containing nonenriched FCSM. Cottonseed meal can be enriched with a maximum of 2% AS before fermentation. Levels of AS up to 2% may not be harmful to fungal fermentation or to performance of growing chicks.

7. The essential amino acids of enriched FCSM might be increased if CSM were also supplemented with starch to provide additional energy. This is proposed because CSM is low in energy and this could be a limiting factor in amino acid synthesis.

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APPENDIX

Table A1. Experiment 1: Analysis of variance of weight gain, feed/gain and feed consumption

Source[a]	d.f.	Mean squares		
		Weight gain	Feed/gain	Feed consumption
Treatment	4	51209.8**	0.353**	349503.56**
C1[b]	1	1682921.0**	1.077**	1015059.6**
C2	1	132016.6**	0.112**	150.0NS[c]
C3	1	226464.5**	0.193**	351122.0**
C4	1	1148.1NS	0.031*	31682.66NS
Error	10	3934.46	0.006	6484.46
C.V., %				

[a]C1 = positive control diets vs all cottonseed meal diets; C2 = soybean meal diet vs supplemented CSM diet; C3 = FCSM diets vs unsupplemented CSM diet; C4 = FCSM - *A. oryzae* diet vs FSCM - *A. janus* diet.

[b]Represents single degree of freedom comparisons between treatment groups.

[c]NS = nonsignificant.

*P < .05.

**P < .01.

Table A2. Experiment 2: Analysis of variance of weight gain, feed/gain and feed consumption

Source[a]	d.f.	Mean squares		
		Weight gain	Feed/gain	Feed consumption
Treatment	4	160697.73**	0.261**	44717.73
C1[b]	1	598291.60	0.781**	116784.04
C2	1	8066.66	0.062**	55296.00
C3	1	33024.5*	0.184**	6086.72
C4	1	3408.17	0.015	704.17
Error	10	5570.40	0.005	20913.6

[a]C1 = positive control diets vs all cottonseed meal diets; C2 = soybean meal diet vs supplemented CSM diet; C3 = FCSM diets vs unsupplemented CSM diet; C4 = FCSM - *A. orvzae* diet vs FCSM - *A. janus* diet.

[b]Represents single degrees of freedom comparisons between treatment groups.

*$P < .05$.

**$P < .01$.

Table A3. Experiment 3: Analysis of variance of weight gain, feed/gain and feed consumption

Source	d.f.	Mean squares		
		Weight gain	Feed/gain	Feed consumption
Treatment	5	22864.18*	0.174**	58976.72**
C1	1	155868.05NS[a]	0.115NS	501.39NS
C2[b,c]	1	68450.00NS	0.009NS	158860.06**
C3[d]	1	163350.00NS	0.111NS	125860.16**
C4	1	720800.22**	0.530**[b]	2312.00NS
C5[e]	1	34352.66NS	0.105NS	7350.00NS
Error	12	56943.27	0.028	11157.61

[a] NS = nonsignificant.

[b] Comparisons of interest.

[c] C2 = supplemented CSM diet vs partially supplemented FCSM diet.

[d] C3 = FCSM - A. oryzae diet vs FCSM - A. janus diet.

[e] C5 = unsupplemented CSM diet vs FCSM - A. oryzae, methionine supplemented.

*P < .05.

**P < .01.

Table A4. Experiment 4: Analysis of variance of weight gain, feed/gain and feed consumption

Source	d.f.	Mean squares		
		Weight gain	Feed/gain	Feed consumption
Treatment	7	151835.42**	0.119**[a]	155546.13NS[b]
C1	1	796797.04**	0.4997**[a]	265651.04NS
C2[a,c]	1	14280.25NS	0.0031NS	20832.11NS
C3[d]	1	1404.50NS	0.0005NS	16501.38NS
C4[e]	1	3504.16NS	0.0043NS	888.16NS
C5[f]	1	106711.11*	0.0003NS	626472.25*
C6	1	118909.38**	0.1755*	1984.50NS
C7	1	21241.50NS	0.1527*	156493.50NS
Error	16	13849.00	0.0233	98101.50

[a]Comparisons of interest.

[b]NS = nonsignificant.

[c]C2 = supplemented CSM diet vs PCSM with 0,1,2% AS.

[d]C3 = FCSM with 0% AS vs FCSM with 1 and 2% AS.

[e]C4 = FCSM with 1% AS vs FCSM with 2% AS.

[f]C5 = unsupplemented CSM vs FCSM with 3,4 and 5% AS.

*P < .05.

**P < .01.

Table A5. Experiment 4: Analysis of variance of level of ammonium sulfate on weight gain

Source	d.f.	Sum of squares	Mean squares
AS	5	634779.61	126955.92**
AS_{linear}	1	517328.23	517328.23**
$AS_{quadratic}$	1	84406.92	84406.92*
Lack of fit	1	33044.46	33044.46NS[a]
Error	12	146058.00	12171.50

[a] NS = nonsignificant.

*P < .05.

**P < .01.